1-1-1980

Salmonella Sp. Harbored By Aquatic Fauna As Indices Of Fecal Pollution

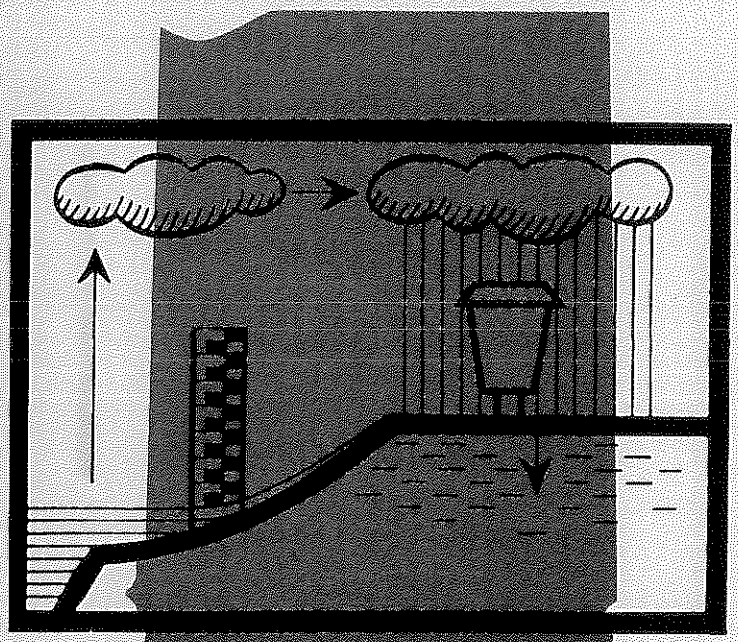
E. V. Morse

K. A. Gossett

R. L. Lawton

TECHNICAL REPORT NO. 127

SALMONELLA sp. HARBORED BY AQUATIC FAUNA AS INDICES OF FECAL POLLUTION



by

Erskine V. Morse
Kent A. Gossett
and
Robert L. Lawton

January 1980

PURDUE UNIVERSITY
WATER RESOURCES RESEARCH CENTER
WEST LAFAYETTE, INDIANA

Water Resources Research Center
Purdue University
Lafayette, Indiana 47907

SALMONELLA SP. HARBORED BY AQUATIC FAUNA AS INDICES OF FECAL POLLUTION

by

Erskine V. Morse
Kent A. Gossett

and

Robert L. Lawton

The work upon which this report is based was supported in part by funds provided by the United States Department of the Interior, Office of Water Research and Technology, as authorized by the Water Resources Research Act of 1974 (PL88-379) as amended.

Period of Investigation: July 1, 1975 - Janaury 1, 1980

Completion Report for OWRT-B-076-IND

Matching Fund Agreement 14-34-0001-6074

Purdue University Water Resources Research Center
Technical Report No. 127
January 1980

ACKNOWLEDGEMENTS

This final report covers various studies conducted under the aegis of Matching Grant Funds OWRT-B-076-IND, and was supported in part by Purdue University. The author and investigators wish to express their thanks to Dr. Dan Wiersma, Director of the Purdue University Water Resources Research Center for his valuable administrative counsel. Dr. Anne Spacie, Department of Forrestry and Natural Resources, Purdue University, gave pertinent advice and assistance in the course of these studies and this is deeply appreciated. Dr. B. Swaminathan, Department of Foods and Nutrition, Purdue University, prepared and provided the special 3MC enrichment broth used in several aspects of the study, and this help is gratefully acknowledged.

The report contains condensed versions of portions of two M.S. degree theses: 1) Kent A. Gossett, D.V.M., "A Study of the Effect of Residence in Fish on the Virulence of Salmonellae in White Mice", Purdue University, May 1978, and 2) Robert L. Lawton, "Transmission of Various Salmonellae Among Fish (Crassius auratus) in the Aquatic Environment", Purdue University, December, 1979. The grant, in part, provided stipend support for these two M.S. candidates during their graduate study.

The valuable technical assistance of Mr. Daniel E. Greenwood, Ms. Edith P. Meyers, Dr. Karl Kersting, and Ms. Marcia J. Kremer is also acknowledged.

TABLE OF CONTENTS

TABLE OF CONTENTS, con't

ABSTRACT FOR ALL SECTIONS OF FINAL REPORT

Techniques for sampling large volumes, i.e. 3 l of river water for salmonellae were developed as suggested by W. H. Ewing (consulting microbiologist, Decatur, Ga., personal communication, 1974). It was possible to ascertain Most Probable Numbers (M.P.N.) for 1 liter samples of river water using concentrated Selenite (Sel) broth. It was established that the common goldfish, Crassius auratus could be experimentally infected with Salmonella when housed in contaminated waters (ca. 4000 l pools).

Eleven serotypes were found among 131 salmonellae isolates when Wabash River (Ind) native fishes and fresh water mussels were cultured. The predominate serotype was S. amsterdam. Salmonellae are capable of colonizing the intestinal tract of native fishes. Higher concentrations of salmonellae were found in fish intestinal tracts than were present in the environmental water. Several serotypes were present in the fish which were not present in their habitat water.

During a three year period, 1973-1976, 833 isolations of salmonellae were made from the Wabash River (Ind) environmenta - ca. 6 km length flow of the river. Thirty-five serotypes were identified. S. typhimurium was the predominant serotype.

Antibiogram profiles of salmonellae of animal origin (160 cultures) versus those isolated from the aquatic environment (637 cultures) were compared. The majority of animal strains were resistant (R) to: ampicillin, chlorotetracycline, streptomycin and sulfonamides. The majority of aquatic isolates were sensitive (S) or intermediate (I) to these same antimicrobials.

Experimental infections in ca. 4000 l pools were established in goldfish with 3 strains of S. typhimurium. Piscine salmonellae infections persisted for 96 days. Fewer salmonellae were demonstrable in the environmental water than were present in the fish intestinal tracts. The same situation existed for native fish in polluted water as indicated above. These data document colonization of the pathogen in fish.

The comparative pathogenicity for mice of various salmonellae serotypes from the native fresh water fishes and their mammalian isolate counterparts was investigated. The serotypes, regardless of source, i.e. host or aquatic environment, had comparable virulence patterns for a given serotype. Smooth (S) variants of an S. tyhpimurium strain retained their virulence after 14, 82, and 96 days residence in fish. When smooth (S) and rough (R) variants of the identical fish strain of S. tyhpimurium were assessed, the S cultures were the more pathogenic. In general, residence in fish did not decrease the virulence of salmonellae for laboratory white mice (NIH-Swiss Webster).

Twelve salmonellae serotypes and their respective 32 strains were all capable of infecting C. auratus. The most pathogenic serotypes, for fish, were S. typhimurium and S. cholerae-suis. Even the host adapted S. tyhpisuis would infect fish for short periods of time.

Increased eutrophy of the water decreased infection rates. However, when bottom sediment was allowed to "build up", increased infection rates were evident.

Physiological stress increased piscine salmonellae infection rates. Since stress is a part of the natural aquatic environment, this fact may play a role in the salmonellae cycle and persistence of the pathogen in contaminated rivers and streams.

Salmonella may be used as monitors of fecal pollution in the aquatic biosphere. The bacteria, when present in natire fishes, may be retrospective indices of fecal pollution. The presence of Salmonella in any body of water raises serious questions as to its safety, i.e. drinking and total body immersion, for both man and livestock.

GENERAL INTRODUCTION

Monitoring fecal pollution of rivers, streams and lakes has been a matter of public controversy and concern for years. A host of bacterial, viral and metazoan parasitic diseases of man and lower animals are transmitted through fecal contamination with water serving as the principal medium. Many of these infections are directly acquired by drinking contaminated water. Fish, mussels, mollusks and other aquatic fauna may serve as intermediate hosts or vectors for infectious agents. Perhaps the most common and easily identified causative agents of enteric disease are bacteria of the genus, Salmonella. There are over 1700 species or serotypes; all of which are pathogenic for both lower animals and human beings. Typhoid (S. typhi), parathyphoid A also C and S. sendai infections are rather strictly limited to man. However, most other serotypes are capable of being transmitted from animals to man and vs. versa. It has been estimated that there may be 2 million cases of human salmonellosis, with approximately 500 deaths, in the U.S. annually. Approximately 1% of our domestic animals and wild fauna may be infected. The vast majority of both human and animal infections are not detected, recognized nor reported. Fortunately, salmonellosis is not a highly fatal disease - except in the very young, the senile and debilitated individuals.

Unequivocally, the presence of Salmonella in the aquatic environment indicates fecal pollution. An actual disease risk and potentiality is, therefore, present when salmonellae are detected. The recognition of salmonellae can be useful and reliable index of contamination of bodies of water used for drinking, bathing, fishing or aquatic recreational activities.

Presently, water quality is judged by total coliform (enteric bacteria) counts, fecal coliform indices and fecal streptococci enumeration. The accuracy, significance and reliability of these microbiologic monitoring parameters have been under question and debate. Progress has been made through technologic advances - new and improved bacteriologic culture media, enumeration techniques, sampling procedures and more sophisticated laboratory tests.

The major areas addressed in the investigations and covered in this report by Sections are:

1. Collection, Sampling and Special Bacteriologic Culture Techn-ques are defined (Section 1 of this report).

2. Presence of Salmonella sp. in the Wabash River and the concurrent harboring of the pathogens in the native fishes in that environment, (Section 2 of this report).

3. Identity of serotypes of salmonellae in river water, river bottom sludge, fishes and river clams, (Section 3 of this report).

4. Antibiograms (antibiotic sensitivities) of salmonellae from the aquatic environment as compared to isolates from cases of salmonellosis in animals,(Section 4 of this report).

5. Pathogenesis of salmonellosis in goldfish (Crassius auratus) and survival of various serotypes of Salmonella in 4465 liter pools, (Section 5 of this report).

6. The virulence of various Salmonella isolated from the aquatic environment was investigated employing the laboratory white mouse as a test animal. The aquatic serotypes were compared with their mammalian host counterparts, (Section 6 of this report).

7. Transmission of various salmonellae serotypes among C. auratus and survival of the enteropathogens in a limited aquatic environment (60 liter aquaria). Comparative virulence evaluations were conducted for various aquatic salmonellae isolates using goldfish as test animals. The importance of stress in piscine infections is evaluated, (Section 7 of this report).

SECTION I

This section delineates special and detailed procedures for the collection of specimens; media preparation for "grant cultures", i.e. 3 liters samples; MPN (most probable number methods); Salmonella identification techniques, and sampling sites employed for fish, clams, bottom sediment and water along the Wabash River. "Giant culture" methodology was employed in sampling the 4465L pools in the initial studies of goldfish infections with Salmonella serotypes.

This section is applicable to the following publications for which the Grant provided partial support:

1. Morse, E.V., Myhrom, E.P. and Greenwood, D.E.: Salmonellosis in Man and Animals as an Environmental Health Problem. Jour. Environ. Sci. and Hlth., All, 755-769. 1976.

2. Morse, E.V. and Duncan, M.A.: Salmonella as Monitors of Fecal Pollution in the Aquatic Environment. Jour. Environ. Sci. and Hlth., All, 591-601. 1976.

3. Morse, E.V., Greenwood, E.D., Meyers, E.P., Anderson, V.L. and Duncan, M.A.: Experimental Salmonella Infections in Crassius auratus (goldfish). Journ. Envir. Sci. and Hlth. All(4), 325-335. 1978.

4. Morse, E.V., Duncan, M.A. and Myhrom, E.P.: Salmonella Serotypes Isolated from the Aquatic Environment (Wabash River, Indiana, 1973-1976). Amer. Jour. Vet. Res., 39, 717-719. 1978.

5. Gossett, K.A.: A Study of the Effect of Residence in Fish on the Virulence of Salmonellae in White Mice. M.S. Thesis, Purdue Univeristy, pg. 52, May, 1979.

6. Lawton, R.L.: Transmission of Various Salmonellae Among Fish (Crassius auratus) in the Aquatic Environment. M.S. Thesis, Purdue University, December 1979.

Collection and Types of Samples:

Aquatic samples, i.e. water, river bottom sludge, fish and fresh water mussels were collected from the Wabash River, West Lafayette, Indiana. Cultures were made from the samples within 3 hours following the collection. The Wabash River and environs with the collection points for water and fish is shown in Figure 1. The two sewage plants employed secondary treatment procedures with continuous chlorination in the case of the Lafayette plant (location E) and the intermittant chlorine treatment at various levels at the West Lafayette Plant (location D).

The following fish were captured by electrostunning:

1) "Gizzard" Shad (Dorosoma cepedianum) bottom filter feeders.
2) Suckers (Moxostoma, sp. and Hypetelium nigrans) insectivorous bottom feeders.
3) Carp (Cyprinus carpio) exclusive bottom feeders.
4) Sauger (Stizostedion canadense) a carnivorous gamefish.

The fresh water mussels were collected manually from a sandy bottom of the Wabash and cultured within 3 hours. The genera represented were: Adondonta and Lampilis. The Collection point was ca. 1.6 km upstream from the Lafayette/West Lafayette metropolitan area (location B). The River at the collection point (B) was "rumored" to be "clean" insofar as sewage was concerned.

Fish gastrointestinal tracts, including livers, were removed and homogenized in sterile Waring blenders. Water samples were collected in sterile containers. Tissues were cultured in Sel and Tet broths at no greater than 10 percent of the total volume of the broth. Water samples were added to sterile concentrated Sel and Tet broths to bring the final concentrations of the inoculated broths up to the recommended volumes (2.3 percent selenite medium and 4.6 percent tetrathionate medium). Serial, 10 fold dilutions of samples were made in sterile 0.85 percent sodium chloride solution. These were agitated on Vortex Genie Mixer R and cultured immediately. Water and sludge samples were passed through Millipore R Filters using GS 0.22μ 47 mm. filter pads; the pads were appropriately divided and cultured in Sel and Tet broths. Results of earlier work by the investigator indicated the use of filters was time consuming and unnecessary, since the methods employed enabled salmonellae isolation from 31 samples of river water. After 24 and again at 48 hours of incubation at 37° broth subcultures were made to one BG agar and to one HE agar plates. The plates were examined after 24 and 48 hours incubation at 37° C for salmonellae-like colonies. The colonies resembling those of the Salmonella were picked to purity plates and subjected to further confirmatory tests.

Methods for Salmonellae Identification:

Biochemical reactions and features for salmonellae identification included those recommended by Ewing, i.e., urease (Christensen urea slants), dectrose, sucrose, and lactose fermentation as well as H_2S production (TSI slants), indol production, methyl red reaction, Voges-Proskauer reaction, citrate utilization, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, dulcitol fermentation, malonate fermentation and motility (Edwards, P.R. and Ewing, W.H.: Identification of the Enterobacteriacae. Burgess Publishing Co., Minneapolis, Minn. 1962, pp. 362). All media were incubated at 37°C for 24-48 hours. Spicer-Edwards serogrouping was performed in the investigator's laboratories using DIFCO antisera. Final serotype indentifications were determined by the Veterinary Services Laboratory, Animal and Plant Health Inspection Service, National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa.

Enumeration of Salmonellae

Estimations of Salmonella numbers in water samples were calculated and based on the five tube method for three dilutions in Standard Methods for Examination of Water and Waste Water, 13th edition, Amer. Pub. Hlth. Assoc., Table 407(2), pg. 673 (1971).

Experimental Salmonellosis in Goldfish (Crassius auratus):

Plastic pools, 3 m in diameter and 0.6 m deep with a capacity of approximately 4465 l, served as the environment for the experiments. The fish were obtained from an Indiana commercial breeder. Approximately 200 fish were placed in each pool which was equipped with a re-circulating water pump (approx. 779 l delivered per hour). The pumps were located so that maximal aeration occurred. The pools were located in 3 separate concrete block houses which had screened windows. Entry of insects, birds and rodents was thus prevented.

Prior to allocating the fish to the 3 experimental pools, the subjects were conditioned for 2 weeks while being maintained in an aerated, flowing water environment consisting of a stainless steel tank approximately 5 x 1.3 x 1 meters. During the period a death loss of ca. 5 percent occurred. The temperature of the conditioning tank ranged from 15-20 C^{O} during the conditioning interval.

Twelve fish were euthanized 14 days prior to infection, and their gastrointestinal tracts removed for bacteriological examination. Total weight of the pooled organs was 22.8 g. The organs were minced with sterile scissors, ground in sterile Pyrex tissue grinders (Belco, 15 ml), divided into four aliquots and cultured in Sel broth and subcultured to BG and HE plating media after 24 and 48 hours incubation as previously described. Abdominal viscera weighing 50 g from 26 fish were examined for salmonellae 7 days prior to infection. Salmonella were not isolated from the fish examined.

Prior to feeding, on two occasions approximately 15 g of feed were cultured in each of 3 flasks of Sel containing 500 ml of broth. Food fed to the fish prior to and during experimentation was negative on culture for salmonellae. The pools were filled with West Lafayette City "tap water" and were allowed to stand for 48 hours "to age" prior to adding the fish. The pumps were started at the time the pools were filled. At the time the fish were added, the water in the 3 experimental pools was negative for salmonellae and did not contain total or free chlorine (Hach Chlorine Test Kit, Model Cn- 66, Ames, Iowa). Water pH was 7.9 - 8.0 and the temperature was 15^{O}C.

FIGURE 1

A = Bridge--US 52 over Wabash River at Lafayette (East side) and West Lafayette, Indiana.

B = West Lafayette, Catherwood Boat Landing.

C = 100 meters/North of East/West R. R. Bridge over the Wabash River.

D = West Lafayette Sewage Plant, Effluent Discharge.

E = Lafayette Sewage Plant, Effluent Discharge.

F = State/Federal National Monument & Recreational Park (West side of Wabash, Fort Quiatenon).

G = Bridge Ind 43--East/West over Wabash River. Sampling point West Half of River.

Figure 1

WABASH RIVER SAMPLING SITES

SCALE=1:125,000

←NORTH

SECTION 2

Presence of *Salmonella sp.* in the Wabash River and the concurrent harboring of the pathogens in native fishes in that environment.

Publication:

Morse, E.V. and Duncan, M.A.: *Salmonella* as Monitors of Fecal Pollution in the Aquatic Environment. Jour. Environ. Science and Health. All, 591-601, 1976.

Abstract

River fish body slime, fish gastrointestinal tracts and fresh water mussels entrails were examined for *Salmonella*. Eleven serotypes were found among 131 isolates. The predominate serotype was *S. amsterdam*. *S. give* and *S. senftenberg* were found only in fish gastrointestinal tracts. Salmonellae are capable of colonizing in fish for periods of time. Their presence is not merely a "filtering out" process. The fish are actually infected and are thus carriers of the salmonellas. *Salmonella*, therefore, may serve as indicators of past and present fecal pollution. As sentinels, they indicate a public health risk to those using the waters for recreation, drinking, etc. The salmonellae water cycle is depicted in Figure 1.

Figure 1

Salmonella in the Aquatic Environment

SALMONELLA IN THE AQUATIC ENVIRONMENT

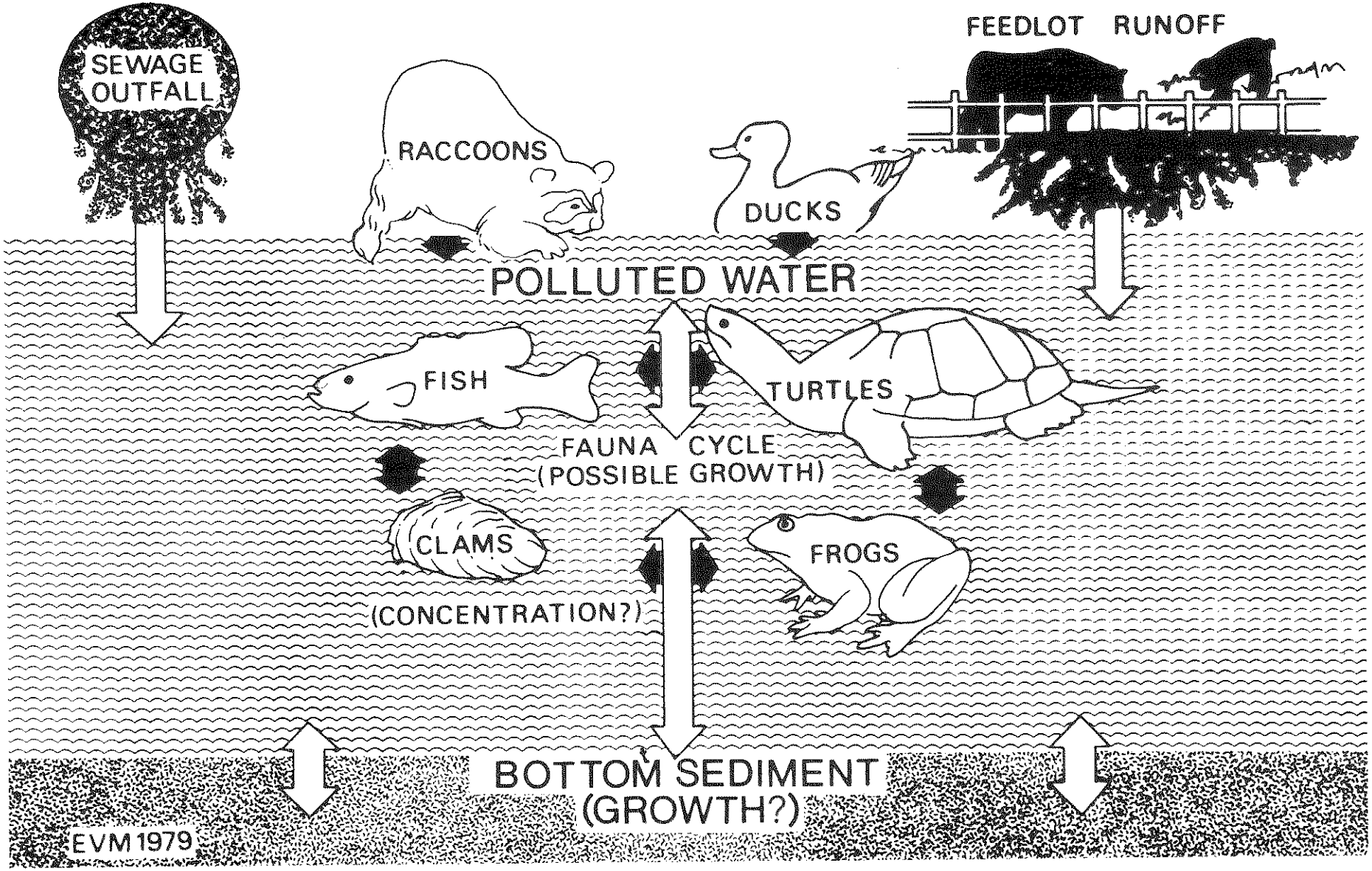


Figure 1

SECTION 3

Identity of serotypes of salmonellae in river water, river bottom sludge, fishes and river mussels (clams).

Publication:

Morse, E. V., Duncan, M. A. and Myhrom, E. P.: *Salmonella* Serotypes Isolated from the Aquatic Environment (Wabash River, Indiana, 1973-1976). Amer. Jour. Vet. Res., 39, 717-719. 1978.

Abstract

During a three year period, 833 isolations of salmonellas were made from the aquatic biosphere - an approximate 6-km flow of the Wabash River above and below West Lafayette/Lafayette, Indiana. Thirty-five serotypes were identified as well as an untypeable O Serogroup C_1. Most isolates were presumed to be of human origin, because of the close proximity of two sewage disposal plants (See Figure 1, Section 1). *S. typhimurium* was the predominating *Salmonella* isolated.

SECTION 4

Comparison of Antibiotic Sensitivities of Salmonella from the Aquatic Environment and those Isolates from Domesticated Animals

Introduction:

The antibiograms (antibiotic sensitivity patterns) of Salmonella isolated from the aquatic biosphere were compated to the pathogens isolated from clinical cases of salmonellosis in domesticated animals. The so called "wild types" i.e., those from water sources, had limited opportunity for wide exposure to the common antibiotics. They would remain sensitive (S) to the antimicrobials.

On the other hand, exposure to antimicrobials at low or high levels could lead to resistance (R). Such, in theory, occurs for salmonellae isolated from domestic animals. Livestock are fed low levels of penicillins, sulfonamides, streptomycin and tetracyclines in their ration as "growth promotants". Domesticated animals receive "shot-gun" antibiotic therapy whenever a disease enters a herd or flock. These two factors favor resistance to antibiotics developing in a salmonellae population.

Coliform bacteria, present in the gut of all chordates, also develop antibiotic resistance by contact with the drugs. This resistance (R-factor) is manifest in the genetic makeup of coliforms. It (R factor) can be transferred to salmonellae. The salmonellas then become resistant to the same antibiotics as were the coliforms.

Many of the antimicrobials which were tested are at this time not used in human therapy, but are used extensively in animal therapies and feeds. Salmonella from human sources are not commonly exposed to tetracyclines, chloramphenicol, ampicillin, polymyxin B, streptomycin, sulfonamides, and neomycin. Since the majority of the aquatic isolates were, in all probability of human origin, it would follow that they should be sensitive to the above drugs.

Materials and Methods:

The Salmonella isolants had been collected during 1972-1977, and represented 160 cultures from clinical cases of mammalian salmonellosis, e.g. 92 equine[1], 52 bovine[2], 9 canine[3] and 7 porcine. Among the equine and bovine strains some cultures were duplicates from the same patient: however, at least 10 days had lapsed between isolations. All the cattle and horses had been hospitalized in the Purdue University Clinic where widespread use of antibiotic therapy was a routine practice[1,2]. Of the 12 antibiotics employed for the antibiogram only nalidixic acid had never been used in therapy.

The Salmonella of aquatic origin were isolated from water (542 cultures) and fish (86 cultures). Fish were captured at points C and E; clams taken at point B, water samples points A-G, and sludge samples at point D (See Figure 1 - Section 1). All samples were obtained from the Wabash river within 9.6 km. of Lafayette, Indiana (Figure 1 - Section 1). The most prevalent serotypes examined and their sources are given in Table 1.

The cultures had been transferred 5 or 6 times on various selective or differential media for identification prior to being placed in storage. Storage cultures were made as deep stabs in sealed tubes of blood agar base (Difco). The cultures were stored in the dark in a laboratory cabinet at approximately 24°C.

Prior to conducting the antibiotic sensitivities, the strains were examined for purity by plating on brilliant green agar (Difco) and Hektoen enteric agar (Difco). Incubation was at 37°C for 24 hours. One or 2 isolated salmonellae colonies were picked from the plates to trypticase soy broth (Balt. Biol. Labs.), incubated at 37°C for 4 to 6 hours at which time the cell count approximated 10^8 per ml.

The Barry, Garcia, Thrupp[4,5] agar overlay modification of the Bauer, et. al.[6] standardized single disc method for antibiotic sensitivity testing was employed. A 0.001 ml calibrated loopful of the broth culture of salmonellae was transferred to 8.0 ml, 1.5 percent aqueous solution of Bacto-agar (Difco), which had been melted and held at least 1 hour at 50°C. The inoculated agar was mixed using a Genie Vortex mixer and spread thoroughly over the surface of a 150 x 15 mm plate of Mueller-Hinton (Difco) agar[7]. The large plate had been held at 37°C for 1-2 hours to facilitate spreading of the agar inoculum. The plates were allowed to stand 5 minutes until the overlay agar hardened. The antibiotic discs were then applied to the surface and gently pressed down with sterile forceps to assure good contact. The plates were inverted, incubated at 37°C for 20-24 hours and the zones of inhibition measured as described by Bauer[6].

Results:

The most prevalent serotypes encountered and used in the antibiogram studies are presented in Table 1. Salmonella typhimurium is the most common cause of salmonellosis in both man and the lower animals. This is apparent in Table 1.

The sensitivity/resistance patterns for the various salmonellae from the aquatic environment and from domestic animals appears in Table 2.

Table 1

Ten Most Prevalent Serotypes Examined and Their Sources

Serotypes	Percent 160 Mammalian Isolates*	Percent 637 Aquatic Isolates**	Percent Total 797 Isolates
typhimurium (both varieties)	45	39	40
anatum	10	0.6	3
enteritidis	4	0.2	1
montevideo	2	2	2
cholerae-suis (both varieties)	2	0	0.5
amsterdam	0	11	9
san-diego	0	8	6
siegburg	0	5	4
saint-paul	0	4	3
give	0	3	2

* 92 equine, 52 bovine, 9 canine and 7 porcine

** 542 river water/bottom sludge, 86 fish and 9 fresh-water mussels

Table 2

Antibiograms of *Salmonella* Isolates From Domesticated Mammals and Aquatic Environment Sources

	Percent 160 Mammalian Isolates			Percent 637 Aquatic Isolates			Percent Total 797 Isolates		
	S	R	I	S	R	I	S	R	I
Ampicillin (10 mcg)	37	61	2(3)*	94	6	1(2)	83	17	1(5)
Chlorotetracycline (30 mcg)	26	53	21	18	7	75	20	16	64
Chloramphenicol (30 mcg)	93	7	--	95	5	--	95	5	--
Furadantin/Macrodantin (300 mcg)	100	--	--	95	4(25)	1(5)	96	4(25)	1(5)
Gentamicin (10 mcg)	89	11	--	100	--	--	98	2(17)	--
Kanamycin 30 mcg)	58	41	1(1)	94	6	--	87	13	1(1)
Nalidixic Acid (30 mcg)	96	1(1)	4(6)	87	1(9)	12	89	1(10)	10
Neomycin (30 mcg)	58	29	13	94	5	1(3)	87	10	3(24)
Novobiocin (30 mcg)	--	100	--	--	100	--	--	100	--
Polymyxin B (300 IU)	100	--	--	100	--	--	100	--	--
Streptomycin (10 mcg)	33	65	2(3)	76	8	16	68	19	13
Triple Sulfonamides (1 mcg)	36	64	--	94	6	1(1)	82	18	1(1)

S = Sensitive at level
R = Resistant at level
I = Intermediate at level
*() number of isolates

References

1. Morse, E. V., Duncan, M. A., Page, E. A., and Fessler, J. F.: Salmonellosis in Equidae: A Study of 23 Cases. Cornell Vet. 66, 198-213, 1976.

2. Morse, E. V., Duncan, M. A., Baker, J. S., Amstutz, H. E., Myhrom, E. P. and Gossett, K. A.: Prevalence, Clinical Aspects, Treatment and Control of Bovine Salmonellosis. Proceed. 8th Ann. Convention, Amer. Assoc. Bovine Pract. 17-20. 1976.

3. Morse, E. V., Duncan, M. A., Estep, D. A., Riggs, W. A. and Blackburn, B. O.: Canine Salmonellosis. A Review and Report of Dog to Child Transmission of Salmonella enteritidis, Amer. Hour, Publ. Hlth. 66, 82, 1976.

4. Barry, A. L., Garcia, F. and Thrupp, L. D.: An Improved Single Disc Method for Testing the Antibiotic Susceptibility of Rapid Growing Pathogens - In: Kirby-Bauer Method for Determining Bacterial Sensitivity. Schering Corporation, Bloomfield, N.J., 07006, 6 pg. leaflet, 1969.

5. Anon: The Kirby-Bauer, Sherris Standardized Single Disc Susceptibility Test for Rapidly Growing Pathogens (Staph. and Enterobacteriaceae). Difco Laboratories, Detroit, Mich., 48232, Technical Information 0348, 1 page. 1971.

6. Bauer, A. W., Kirby, W. M. M., Sherris, J. C. and Turck, M.: Antibiotic Sensitivity Testing by a Standardized Single Disc Method. Amer. Jour. Clin. Path., 45, 493-496, 1966.

7. Barry, A. L.: The Routine Antibiotic Disc-Plate Sensitivity Tests. 1. Variations in the Size of Inoculum. Amer. Jour. Med. Tech., 30, 153-161, 1964.

SECTION 5

Pathogenesis of salmonellosis in goldfish (Crassius auratus) and survival of various serotypes of Salmonella in 4465 L pools.

Publication:

Morse, E. V., Greenwood, D. E., Myers, E. P., Anderson, V. L. and Duncan, M. A.: Experimental Salmonella Infections in Crassius auratus (Goldfish). Jour. Environ. Sci. and Health. A13(4), 325-335. 1978.

Abstract

The common goldfish has been experimentally infected with S. typhimurium, isolated from Wabash River fishes and S. typhimurium, variant Copenhagen cultured from a dead pig. Piscine infections lasted as long as 91 days. The bacteria were able to colonize in the fish gut and their multiplication occurred. Fewer salmonellae were present in the environmental water than were present in the fish entrails.

The investigator and project personnel are shown (Figure 1) removing fish from the experimental pool.

FIGURE 1

Investigators capturing uninoculated fish from stock pool for transfer to inoculated (S. typhimurium) pool.

Figure 1

SECTION 6

Effect of Residence in Fish on the Virulence of *Salmonella* for White Mice

Introduction:

Transmission of salmonellosis between man and animals may readily occur; however, the role of fish and other aquatic fauna in the epidemiology and epizootiology of salmonellosis remains obscure. Fish and fish products have been known to be contaminated with salmonellae.[2,5,8,12] Increasing evidence indicates that fish may be naturally [9,11,13,14] and experimentally infected [1,9] with the various serotypes of the genus, *Salmonella*. Pathological changes characteristic of *Salmonella* infection have been reported and fish have been shown to develop serological titers for the pathogens.[1,3] These several studies incriminate fish as possible vectors of human and animal salmonellosis.

In this investigation, infection was initiated in white mice with fish isolates and with cultures of the same serotype obtained from clinical cases of human and lower animal salmonellosis. Laboratory white mice were injected intraperitoneally with serial dilutions of salmonellae broth cultures prepared from two or more isolates of the same serotype. LD_{50} and ID_{50} values were calculated. Results of these trials were compared to detect differences in virulence of the isolates.

The goal of this study was to determine if Salmonella organisms are attenuated by residence in fish. Results could indicate the potential salmonellosis hazard that infected fish might have for man and other animals.

Materials and Methods:

Cultures:

Fish isolates were selected from among the 833 salmonellae isolated from aquatic environmental samples by the author during 1973-1976. Samples were collected along a 7 mile flow of the Wabash River through Lafayette and West Lafayette, Indiana. Isolates selected for this study were recovered from the alimentary tracts, including liver, of "gizzard shad" (*Dorosoma cepedianum*), suckers (*Moxostoma ssp.* or *Hypetelium nigrans*), and carp (*Cyprinus carpio*). These cultures are listed in Table 1.

Table 1

Serotypes isolated from fish collected from the Wabash River near Lafayette, Indiana.

Serotype*	Date of Isolation
S. senftenberg	8/5/74
S. montevideo	10/8/73
S. typhimurium	7/28/75
S. amsterdam	10/8/73
S. amsterdam	8/5/74
S. give	8/5/74
S. newington	7/28/75

* Final serotype indentification of all cultures was determined by Dr. Billie O. Blackburn, Veterinary Service Laboratory, Animal and Plant Health Inspection Service, National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa.

Salmonella from animal and human sources were obtained from the National Animal Disease Center (NADC), Ames, Iowa, and the Indiana State Board of Health, Indianapolis, Indiana, respectively. These serotypes are listed in Table 2.

Table 2

Animal and human isolates obtained from National Animal Disease Center (NADC) and Indiana State Board of Health (SBH), Respectively.

Serotype	Identification Code	Source
S. senftenberg	SBH E 1-2 (4/23/76)	Human
S. montevideo	SBH E 1-64 (12/5/74)	Human
S. amsterdam	NADC #4	Poultry
S. give	NADC 76-3151	Poultry
S. newington	NADC 76-3159	Equine

The source and identity of cultures used in special comparative studies of virulence are given in Tables 3-5.

Table 3

Cultures obtained from studies of experimental *Salmonella* infection in goldfish (*Crassius auratus*) with a strain of *S. typhimurium* isolated from fish from the Wabash River on 7/28/75.

Identification Code	Culture Description
F	originally isolated from fish collected from Wabash River on 7/28/75 (16B)
F14	recovered from goldfish 14 days after experimental infection with culture F (16B #2A 14 days)
F82	recovered from goldfish 82 days after experimental infection with culture F (16B 82 days)
F96	recovered from goldfish 96 days after experimental infection with culture F (16B 96 days)

Table 4

Cultures obtained from studies of experimental *Salmonella* infection in goldfish (*C. auratus*) with a strain of *S. typhimurium* var. copenhagen isolated from swine.

Identification Code	Culture Description
P	originally isolated from a pig (Rodruck)
P14	recovered from goldfish 14 days after experimental infection with culture P (Rod Fish #7A 14 days)
P68	recovered from goldfish 86 days after experimental infection with culture P (Rod Fish 68 days)

Table 5

Cultures obtained from studies of experimental *Salmonella* infection in goldfish (C. auratus) with a strain of *S. typhimurium* var. copenhagen isolated from a human.

Identification Code	Culture Description
H	originally isolated from a human (Talk XS_9) Smooth (S) phase.
H6#1	recovered from goldfish 6 days after experimental infection with culture H and found to be a rough (R) culture (AR_7)
H6#2	recovered from goldfish 6 days after experimental infection with culture H and found to be a rough (R) culture (CR_7)
H27	recovered from goldfish 27 days after experimental infection with culture H and found to be a smooth (S) culture (BS_6A)

Criteria for establishing the smooth (S) - rough (R) status of the cultures were: reactions in normal and Salmonella specific rabbit sera, growth in broth cultures, colonial morphology as viewed with a dissecting, stereoscope (10x) with obliquely transmitted light, and the reaction (agglutination) in 1:5000 neutral acriflavin.

Media and Bacterial Identification:

Media, *Salmonella* typing sera and reagents were prepared according to manufacture's directions. Incubation of media was at 37C. All media were obtained from Difco Laboratories except Trypticase Soy Broth (TSB) which was manufactured by Baltimore Biological Laboratories.

Salmonellae Infecting Inocula Preparation:

Three brilliant green (BG) plates were streaked from the cultures to be compared. After 24 hours incubation, the plates were examined under the stereoscope, as previously described, and five isolated, smooth appearing colonies were picked and inoculated into 100 ml Trypticase Soy Broth (TSB). The TSB culture was incubated for 4-6 hours until the bacterial suspension was moderately turbid. The broth culture was diluted with sterile 0.85% saline to attain a visual density

equivalent to MacFarland standard tube #1 ($3x10^8$ organisms per ml). This standardized tube served as the index point for tenfold serial dilutions through $1:10^8$. The number of bacteria per ml was determined by plating 0.1 ml of the last (highest) four dilution tubes on quadruplicate tryptose agar plates (TSP). Even distribution of the bacteria on the plates was accomplished by using sterile glass spreaders (hockey sticks).

Groups of six mice (three male and three female) each received intraperitoneal injections of 1 ml of the respective dilution. The inoculation procedure was repeated for all dilutions to be tested.

Experimental Animals:

NIH-Swiss Webster mice (Murphy Laboratories, Plainfield, Indiana), weighing 20-30g served as experimental animals. Equal numbers of males and females were used. Three mice of the same sex were housed in each of two plastic box cages (7½ x 11½ x 5 inches) with a wire cover. Test groups consisted of one cage of each sex contained in a modified Horsfall isolation unit for each dilution of inoculum. Wayne Lablox mouse diet and West Lafayette tap water were given ad lib. Feed, water and bedding were cultured prior to the start of the experiments and were found to be consistently negative for salmonellae. The supplier's mouse colony was known to be free of salmonellosis. However, pooled samples of liver, spleen and colon of each of 6 mice from each shipment were cultured for salmonellae. All controls were found to be negative. In addition, six uninoculated mice were housed in the same room as the inoculated mice as controls.

Pathogenicity of the salmonellae was ascertained by calculation of the lethal dose in 50% of the test animals (LD_{50}) and the infective dose after 6 days in 50% of the mice (ID_{50}). The method of Reed and Muench was applied to the data for these calculations. *

Isolation of Salmonellae from the Mice:

Six days following inoculation all surviving mice were sacrificed. The animals were killed by cervical disarticulation, placed in dorsal recumbancy, their abdomens swabbed with 70% ethanol and opened with sterial instruments.

All mice which died and those which survived for 6 days were examined bacteriologically. Approximately 0.5g of liver and spleen were aseptically removed to a sterile petri dish, minced and a standard loopful streaked on brilliant green agar plates (BG). A section of colon was excised and minced with the remaining liver-spleen admixture and inoculated into tubes (18 x 150 mm) containing 15ml of selenite broth (Sel). After 24 hours of incubation the direct liver-spleen BG cultures were examined for the presence of salmonellae. The Sel cultures were streaked on BG plates, incubated for 24 hours, and then examined. Colonies resembling salmonellae were transferred to triple sugar iron agar slants (TSI) and Christensen's urea slants, incubated for 24 hours and examined for characteristic reactions. In addition, positive cultures were confirmed by agglutination in a polyvalent flagellar antiserum (1:100 dilution) tube test.

*Reed, L.J., Muench, H.: A Simple Method of Estimating 50% Endpoints, Amer. J. Hyg., 27(3):493-497, 1938.

Results:

All control animals were culturally negative for salmonellae throughout these experiments. Periodic checks of feed, water, and bedding supplies were also free of Salmonella.

S. typhimurium recovered from fish collected from the Wabash River (Culture F), S. typhimurium var. copenhagen of swine origin (Culture P), and S. typhimurium var. copenhagen of human origin (Culture H) were selected for repeat trials. Summary of these results is found in Tables 6-8. Culture F and LD_{50} values of 9.00×10^4 and 1.51×10^4 on test groups that were similar except that they were not run concurrently. Culture P and LD_{50} values of 5.48×10^3 and 6.70×10^3. Culture H had LD_{50} values of 1.45×10^1 and 1.35×10^2.

Experimental infection of goldfish with S. typhimurium (Culture F--originally recovered from fish captured from the Wabash River) yielded mixed results (Table 6). Isolate F14 (recovered 14 days after experimental goldfish infection) showed a decrease in lethality for mice. However, later goldfish isolates F82 (82 days) and F96 (96 days) indicated no decrease in the ability to cause death in mice had occurred for this strain after a second passage through C. auratus.

Table 6

Tabulated results of LD_{50} calculations for comparison of cultures obtained from experimental infection of goldfish (C. auratus) with S. typhimurium originally isolated from fish (Culture F).*

Culture	LD_{50}
F (original isolate from Wabash River fish)	9.00×10^4
F (original isolate from Wabash River fish--replicate trial)	1.51×10^4
F14 (14 days in goldfish)	29.8×10^4
F82 (82 days in goldfish)	1.35×10^4
F96 (96 days in goldfish)	3.14×10^4

* See Table 3 for more complete culture description.

Results of the S. typhimurium var. copenhagen originally from swine (Culture P) were more uniform (Table 7). P14 (re-isolated 14 days after experimental infection of fish) and P68 (68 days) had no decrease when compared with the parent strain.

Table 7.

Tabulated results of LD_{50} calculations for comparison of cultures obtained from experimental infection of goldfish (C. auratus) with S. typhimurium var. copenhagen originally isolated from swine (Culture P).*

Culture	LD_{50}
P (original isolate from swine)	5.48×10^3
P (original isolate from swine-- replicate trial)	6.70×10^3
P14 (14 days in goldfish)	6.77×10^3
P68 (68 days in goldfish)	1.33×10^3

* See Table 4 for more complete cultural description

Results of mouse infection with S. typhimurium var. copenhagen of human origin (Culture H) before and after goldfish passage are summarized in Table 8. LD_{50} values for this trial varied as much as 10^6. The R mutants were less virulent than their S phase counterparts.

Table 8.

Tabulated results of LD_{50} calculations for comparison of cultures obtained from experimental infection of goldfish (C. auratus) with S. typhimurium var. copenhagen originally isolated from human (Culture H).*

Culture	LD_{50}
H (originally isolated from human) S phase culture	1.45×10^{1}
H (originally isolated from human-replicate trail conducted 4 months later) S phase culture	1.35×10^{2}
H6#1 (isolated in Rough phase after 6 days in goldfish)	5.7×10^{6}
H6#2 (isolated in Rough phase after 6 days in goldfish)	1.39×10^{7}
H27 (isolated in Smooth phase after 27 days in goldfish)	2.51×10^{4}

* See Table 5 for more complete culture description

A marked difference was seen in the mortality produced by S. typhimurium (Culture F) and the two strains of S. typhimurium var. copenhagen (Cultures H and P). Culture F was less lethal than either of the S. typhimurium var. copenhagen strains. Variation in lethality also was detected between the two strains of S. typhimurium var. copenhagen.

Serotypes senftenberg, montevidio, amsterdam, give, and newington were all less virulent for mice than were S. typhimurium and its variant copenhagen. There were also differences in virulence between serotypes senftenberg, montevideo, amsterdam, give, and newington. LD_{50} values ranged from 2.31×10^{7} to 25.5×10^{7} and ID_{50} values were between 5.51×10^{2} and 3.16×10^{4}. However, all serotypes in this group (senftenberg, montevideo, amsterdam, give, and newington) showed close correlation between virulence of the cultures within the same serotypes, whether they originated from fish or warm-blooded animals. These data are shown in Table 9.

Table 9.

Tabulated results of LD_{50} and ID_{50} calculations for comparison of Wabash River fish isolated with animal isolates.*

	LD_{50}	LD_{50}
S. senftenberg		
Fish 8/5/74 #4	2.43×10^{7}	$1.38 c 10^{4}$
Human 4/23/76 SBH E1-2	2.31×10^{7}	3.15×10^{4}
S. montevideo		
Fish 10/8/73 #21	3.95×10^{7}	5.51×10^{2}
Human 12/5/74 SBH E1-64	2.45×10^{7}	19.7×10^{2}
S. amsterdam		
Fish 10/8/73 #6	9.35×10^{7}	2.35×10^{3}
Fish 8/5/74 #20	10.8×10^{7}	4.84×10^{3}
Chicken NADC #4	5.61×10^{7}	2.53×10^{3}
S. give		
Fish 8/5/74 #74	10.9×10^{7}	5.46×10^{3}
Chicken NADC 76-3151	4.85×10^{7}	2.62×10^{3}
S. newington		
Fish 7/28/75 #23B	9.11×10^{7}	3.29×10^{3}
Horse 76-3159	25.5×10^{7}	1.79×10^{3}

*All fish isolates from author's previous investigations.
SBH - Indiana State Board of Health culture
NADC - National Animal Disease Laboratory (USDA), Ames, Iowa

Discussion:

Many investigators have published on infection of mice with S. typhimurim. The virulence pattern and course of the disease resembled those of S. typhi infection (typhoid fever) in man. The virulence for mice of the other serotypes in this study (senftenberg, montevideo, amsterdam, give, and newington) has not been reported in the literature to the knowledge of the investigator.

With the exception of work by Brunner,[1] no other studies dealing with experimental murine infections with Salmonella of fish origin could be found. She demonstrated that S. enteritidis isolated from fish several weeks after experimental infection showed no impairment of virulence for mice.[1]

Repeat trials on the parent strains of Cultures F, P, and H were conducted to test reproducibility of data (Tables 6-8). There was close correlation between Culture P results, while a difference of almost one log was determined for culture H. Culture F repeat trails showed variation intermediate between Culture P and Culture H.

Larson, et.al. conducted parallel and concurrent titrations of identical endotoxin preparations and found a variability in the lethal dose of greater than 100%. They considered this variation not to be extreme.[6] Due to the probability of smooth-rough transformation (S-R) in Culture H, which will be discussed later, the difference in LD_{50} values for these repeat trails was considered too large to be attributed to normal variation of equally virulent strains. Culture F showed some variation on repeat trials. However, results of mouse inoculation with cultures from fish experimental infections, exhibited no trend toward change in virulence. This indicates that the differences seen in the LD_{50} on repeat trials of the parent Culture F can probably be attributed to normal variation of equally virulent strains. For the purpose of this paper, difference in virulence less than that experienced in Culture F repeat trails (0.6 log) was considered normal experimental variation for equally virulent strains.

Serotypes senftenberg, montevideo, amsterdam, give, and newington showed good correlation between virulence of cultures within the same serotype, i.e. variation less than 0.6 log. This suggests that no significant attenuation of the pathogen occurred as a result of residence in a poikelothermic host.

Inspite of the significant difference in virulence among some of the members of the serotypes senftenberg, montevideo, amsterdam, give, and newington, all LD_{50} values were between 2.31×10^7 and 25.5×10^7 and ID_{50} values were between 5.51×10^2 and 3.16×10^4. These serotypes were all less virulent than the smooth strains of S. typhimurium and its variant copenhagen. It is hypothesized that if more of the serotypes "not host adapted" to mice (excluding S. typhimurium) were tested, the results would be similar to those found in this study.

Strains of the same serotype with different virulences have been reported by other authors[4,10] and the present trials of S. typhimurium and its variant copenhagen reaffirms their findings.

Results of the Culture F and Culture P trials showed no trend toward decrease in virulence occurred after experimental fish passage (Tables 6-7).

S. typhimurium var. copenhagen (Culture H) originally from a human case of clinical salmonellosis was used to experimentally infect fish. After 6 days of infection, isolations from fish had mutated from smooth (S) to rough (R) phase. Two of these isolates, H6#1 and H6#2, satisfied the criteria of the R variant, i.e., form granular precipitate in broth culture, had matt surface colonial morphology, agglutinated in normal non-specific sera, and agglutinated in the presence of neutral 1:5000 acriflavin. Another isolated remained in the smooth form after 27 days in fish (H27). These three cultures and the parent culture were used for experimental mouse infection. Results of these trials are listed in Table 8. All three of the fish isolates had a decrease in lethality when compared with the parent S strains. R variants of Salmonella have been shown to be less virulent than their parent S strains.[4] Isolates H6#1 and H6#2 reaffirm the work of these authors. Culture H27, which was determined to be smooth phase, also had a decrease in lethality. This could be explained by recognizing that the transformation from smooth to rough phase (S-R) may be a gradual rather than one step processn and that there are varying degrees of roughness. It is hypothesized that the original human isolate (Culture H) was in a state of transition from S to R phase or contained mutant forms. There was even a decrease in lethality between repeat trails of the parent strain that were conducted four months apart. It is possible that H27 may represent an intermediate form (I) in the S-R transformation and therefore exhibits intermediate lethality.

S. typhimurium var. copenhagen (Culture H) mutated rapidly to the R phase after fish inoculation. Procedures for the three experimental fish infections cited were similar with the exception of environmental (Water) temperatures. Temperatures for the cultures F and P infections in fish ranged from 15-22°C. If the speed of the S-R transformation is enhanced by the lower temperature and is reproducible, this would suggest that the lower temperature may cause selective pressure for rough variants within cold-blooded hosts.

REFERENCES

1. Brunner, G.H.: Experiments on the Possibilities and Course of Infection with Salmonella enteritidis and Salmonella typhimurium in Freshwater Fish, Zbtl. Bakt. Hyg. I Abt., Orig. B, 158:412-431, 1974.

2. Gulasekharam, J., Velaudapillae, T., Niles, G.R.: The Isolation of Salmonella Organisms From Fresh Fish Sold in Colombo Fish Market, J. Hyg., 54:581-584, 1956.

3. Janssen, W.A., Meyers, C.D.: Fish: Serologic Evidence of Infection with Human Pathogens, Science, 159:547-548, 1968.

4. Jarolmen, H., Kemp, G.: Association of Increased Recipient Ability for R Factors and Decreased Virulence Among Variants of Salmonella choleraesuis var. kunzendorf, J. Bact. 97:962-963, 1969.

5. Kleeman, I., Frant, S., Abrahamson, A.E.: Food Poisoning Outbreaks Involving Smoked Fish--Their Epidemiology and Control, Amer. J. Public Health, 32:151-158, 1942.

6. Larson, C.L.E., Ribi, E., Milner, K.C., Lieberman, J.E.: A Method of Titrating Endotoxic Activity in the Skin of Rabbits, J. Exptl. Med., 111:1-20, 1960.

7. Lewis, D.H.: Retention of Salmonella typhimurium by Certain Species of Fish and Shrimp, J. Amer. Vet. Med. Assn., 167:551-552, 1975.

8. Mackenzie, C.R., Livingstone, D.J.: Salmonellae in Fish and Food, S. Afr. Med. J., 42:999-1003, 5 Oct 68.

9. Martin, W.T.: Occurrence and Survival of Salmonellae in the Alimentary Tract of Some Freshwater Fishes, Masters Thesis, Kansas State Univ., Manhatten, Kansas.

10. McCulloch, N.B., Eisele, C.W.: Experimental Human Salmonellosis I, Pathogenicity for Strains of Salmonella meleagridis and Salmonella anatum Obtained from Spray-dried Whole Egg, J. Inf. Dis., 88:278-289, 1951.

11. Morse, E.V., Duncan, M.A., Myhrom, E.P.: Salmonella Serotypes Isolated From the Aquatic Environment (Wabash River, Indiana, 1973-1976), Am. J. Vet. Res., April, 1978, (in press).

12. Shervan, J.M., Liston, J.: A Review of Food Poisoning Caused by Fish and Fishery Products, J. Appl. Bact., 18:522-534, 1955.

13. Souter, B.W., Sonstegard, R.A., McDermott, L.A.: Enteric Bacteria in Carp (Cyprinus carpio) and White Suckers (Catostomus commersoni), J. Fish Res. Board Canada, 33:1401-1403.

14. Trawinska, J.: Examination of the Microflora of the Alimentary Canal of Pond Fishes for the Presence of Salmonellae, Med. Weterinaryjna, 5(3):184-187. Cited from Biol. Abst., 24, Number 20538.

SECTION 7

Transmission of Various *Salmonella* and Their Respective Strains Among Goldfish (*Crassius auratus*).

Introduction:

The presence of *Salmonella* in the aquatic environment and the appraisal of the significance of such a finding is complicated as a result of two possible sources contributing to the continued survival of *Salmonella* in freshwater. Evidence from Hendricks[1,2] and Morse[3] has demonstrated in rivers, streams or lakes that both numbers and serotypes of *Salmonella* are greater in the bottom sediments than in the water overlay. Additional evidence suggests that the microorganisms may even multiply in bottom sludge.[4-8] Viscera of freshwater fish and fauna have been shown to contain *Salmonella*.[9-16] If the bacteria are capable of surviving, concentrating or multiplying in fish viscera, resultant water pollution may further be accentuated via fecal excretion or shedding of salmonellae from infected fish.[12-15]

Martin isolated *Salmonella* from experimental infection of channel catfish (*Ictalurus punctatus*) for 29 days[13] and Lewis for 30 days.[16] Morse, et al., isolated *S. typhimurium* from water containing experimentally infected goldfish (*C. auratus*) for 188 days.[3] Brunner isolated salmonellae from trench (*Tinca tinca*) for 60 days following experimental infection.[12] Increased duration of residency was correlated with increased water temperatures (16-17°C).[12] *S. enteritidis* was isolated from carp (*Cyprinus carpio*) 110 days following experimental infection and a "pseudomembranous inflammation" of the intestine was reported.[12] Experimental infection of goldfish with *S. typhimurium* produced "enteric alterations similar to those observed for salmonellosis in higher animals."[3] This fact coupled with a two log increase in bacterial density in fish viscera as compared to the fishes' environmental water suggested that the pathogens had colonized in the fish gut.[3]

Presence and duration of salmonellae infection in fish may be precipitated by age, stress factors, and environmental water conditions. Duration may also be a function of the concurrence of other infections; the species of fish involved; the fishes' breeding status; and seasonal changes. The complexitites of reoccurring *Salmonella* isolations from the aquatic environment warrants further investigation and elucidation. The purpose of this research was directed towards (1) analysis of the transmission patterns for 12 serotypes and 32 strains respectively of these *Salmonella* to *C. auratus* (goldfish), (2) the duration of survival in water of various serotypes/strains and (3) serotype infectivity for goldfish as related to various controlled, environmental parameters.

Materials and Methods

Cultures:

Twelve serotypes representing 32 strains of *Salmonella* were used in this study. These serotypes, respective strains and origins are listed in Table 1.

Table 1
Culture Collection

Serotype	Strain	Origin
agona	400	Isolation from Wabash River Basin (Morse) 3/7/76
agona	400-R_1	Isolation from fish passage of S. agona 400 for 7 days (Lawton) 11/10/78
agona	400-R_2	Isolation from fish passage of S. agona 400-R_1 for 7 days (Lawton) 11/20/78
agona	400-R_3	Isolation from fish passage of S. agona 400-R_2 for 7 days (Lawton) 12/5/78
agona	400-R_4	Isolation from fish passage of S. agona 400-R_3 for 7 days (Lawton) 12/18/78
anatum	RH_2O #17	Isolation from Wabash River Basin (Morse) 12/5/73
anatum	200	Isolation from horse 11/13/73
eimsbuettel	789	From CDC[a] 12/4/74
enteritidis	NAR	From G.H. Snoeyenbos, Univ. of Massachusetts, Amherst. Resistance to nalidixic acid 500 ug/m[b]
enteritidis	35	Fish isolate, Wabash River (Morse) 5/3/76
enteritidis	Ostenberg	Dog to human transfer isolate[c] 1/7/74
derby	15-4(21)	From CDC 12/4/74
infantis	21	Isolation from Wabash River Basin, Big Colony Type (Morse) 6/24/75
montivideo	623C467	From CDC 12/4/74
typhimurium	24RH_2O	Naturally nalidixic acid resistant at 500 ug/ml strain Wabash River (Morse) 5/19/75
typhimurium	Lucus	Human isolation (Morse) 12/5/73
typhimurium var. Copenhagen	Rodruck	Isolation from porcine feces (Morse) 4/26/73

Table 1, cont.

Serotype	Strain	Origin
typhimurium var. Copenhagen	Talkington	Horse to human transmission,[d] human isolate (Morse) 6/20/76
typhisuis	3198	NADC[e]
typhisuis	1784	NADC
typhisuis	2637	NADC
typhisuis	1342	
cholerae-suis	164	NADC
cholerae-suis	1102	NADC
cholerae-suis	2992	NADC
cholerae-suis	1103	NADC
cholerae-suis var. Kunzendorf	3648	NADC
cholerae-suis var. Kunzendorf	3094	NADC
cholerae-suis var. Kunzendorf	3520	NADC
cholerae-suis var. Kunzendorf	Local PIG H_2S^+	Naturally infected pig. (Morse) 1975.

[a] Center for Disease Control, Atlanta, Georgia.

[b] Nalidixic acid resistance, laboratory developed.

[c] Morse, E.V., Duncan, M.A., Estep, D.A., Riggs, W.A., Blackburn, B.O.: Canine Salmonellosis: A Review and Report of Dog to Child Transmission of Salmonella enteritidis, AJPH, 66:1:82-84, 1976.

[d] Morse, E.V., Kersting, K.W., Smith, L.E., Myhrom, E.P., Greenwood, D.E.: Salmonellosis: Probable Transmission from Horse to Human to Dog of Infection, AJPH, 68:5:497-499, 1978.

[e] National Animal Disease Center, APHIS, U.S. Dept. of Agriculture, Ames, Iowa.

Media Employed:

All stock cultures were maintained on Nutrient Agar. Selenite Broth (Sel) was routinely used as a selective/enrichment medium with subcultures made onto Brilliant Breen (Bg) and Hektoen Enteric (He) agar plates. All

these media were obtained from DIFCO laboratories and were prepared according to their directions. Nalidixic acid resistant (NAR) strains of S. tyhpisuis were developed by repeatedly culturing the stocks in Tripticase Soy Broth (TSB) (Baltimore Biological Laboratory, BBL, Baltimore, MD) containing 100, 250, and 500/ug/ml nalidixic acid. All inoculated media were incubated at 37°C.

Cultures used as inocula were grown in TBS for 7-24 hours depending on the serotype to be used. They were serially diluted in a 1 percent solution of Bacto-Peptone (DIFCO). Direct plating was made onto Tryptic Soy Agar plates (TSA) (DIFCO). Uniform dispersion of 0.1 ml of the inoculum on the agar surface was accomplished by the use of sterile glass "hockey stick" spreaders while the plates were rotated rapidly. Colony forming units (CFU) were counted employing a Quebec Colony Counter, and thus the number of salmonellae cells in the dilution ascertained.

For growth and media comparison studies, Tetrathionate Broth (Tet), 3MC broth (3MC is a special broth media developed by Dr. B. Swaminathan, Purdue University), and Sel were used. The 3MC media was utilized for experimentation with S. cholerae-suis, both varieites, as well as S. typhisuis. These serotypes were not inhibited in 3MC medium as they were in Sel or Tet broths. Standard biochemical testing media (DIFCO) were employed as recommended by Ewing (Edwards, P.R., Ewing, W.H.: Identification of Enterobacteriaceae, 3rd edition, 1972, Burgess Pub. Co., Minneapolis, Minn.).

Experimental Animals Used:

Three to four inch Crassius auratus, common goldfish, were obtained from Grassy Forks Fisheries, Martinsville, Indiana, for this study. They were housed in plastic, 4200 liter pools containing aged, West Lafayette, tap water until used in the infectivity trials. Water temperatures ranged between 65° and 80°F. Fish were fed antibiotic-free, pelleted feed obtained from Grassy Forks Fishery. The feed was ascertained to be salmonellae-free as was the water housing experimental fish as well as the purchased fish.

Preparation of Inocula and Challenge Procedures:

Stock cultures of the serotypes for inoculation were planted onto Bg and He agar plates and incubated for 24 hours at 37°C. The criteria employed to determine smooth (S) or rough (R) phase cultures included typical colonial morphology as viewed under 10x power with a dissecting-type microscope and fialure of cultures to agglutinate in 1:5000 neutral acraflavine. Final identification of the Salmonella was accomplished by inoculation of biochemical media, i.e. Triple Sugar Iron (TSI), Christensen Urea Agar (urea) and Lysine Iron Agar (LIA) slants (DIFCO). Cultures conforming to criteria typical for salmonellae were further confirmed through appropriate serological reactions with specific O Group antisera (agglutination) and with normal rabbit sera (DIFCO) (nonagglutination) using the macroscopic slide test.

Ten isolated colonies were picked from either Bg or He plates and inoculated into 100 ml TSB. After 7 to 8 hours incubation at 37°C, the cultures were serially diluted ten fold, 10^1 through 10^8 and plate counts were made of the three highest dilutions, i.e. 10^{-6} -10^{-8} and plate counts were made of the three highest dilutions, i.e. 10^{-6} -10^{-8}, on TSA plates. One ml of the dilution containing 10^6 organisms (CFU) was then added to 60 liters of water in each of the experimental tanks. These tanks were 75 liter, "show-type," glass aquaria with dimensions of 61 cm, by 31 cm, by 42 cm. A final concentration of 100-200 salmonellae/ml was established.

Studies to ascertain the levels of infectivity of various Salmonella serotypes were undertaken. Duplicate trials were conducted for each dilution of 10^2 -10^6 salmonellae/ml in the final water concentration. S. typhisuis (NAR) strain inoculum levels were 10^5 cells/ml of tank water. This level was necessary to produce infection based on preliminary data.

Experimental Paramsters and Monitoring Procedures:

Estimates of Salmonella numbers in aquaria water were determined at the time of inoculation, 7 days and at 14 days following inoculation using the Most Probable Number (MPN) technique, i.e. 5-tube, 3 fold dilution method (Standard Methods for the hxamination of Water and Wastewater, 13th ed., American Public Health Assoc., 1971, pp. 664-68).

Adequate oxygenation (8-10 mg/l DO) was provided with a circulation pump (Little Giant Model 1) delivering a flow of 7.5/1 hr which was placed at the bottom of each tank (Dissolved Ocygen Test Kit, Model OX-ZP, Cat. No. 1469-99, Hach Chemical Co., Ames, Iowa).

Necropsy and Culture Procedures:

Fish were anesthisized by packing in ice. They were pithed with sterile forceps prior to immersion in 70% ethanol for 1-2 minutes. Repeated culture of skin swabs before and after the alcohol bath (fish dried with sterile paper towels) yielded Salmonella from the pre-alcohol bath swabs only.

The abdomen of each fish was opened, the viscera excised, placed in a sterile petri dish, and minced with sterile instruments. The minced organs, i.e. gastrointestinal tract and liver, were plated directly onto Bg plates. The remaining inocula, 1-5 grams, were transferred to 150 ml Sel or 3MC broth, contained in Delong Flasks and incubated at 37°C. Subcultures to Bg were made at 24 and 48 hours. Purity cultures were made by streaking suspect colonies to Bg, and confirmed as Salmonella by appropriate reactions on TSI, urea and LIA slants. Further proof was obtained by agglutination in polyvalent and specific Salmonella somatic (0 Group) antisera.

Five uninoculated fish served as controls for each trial. Their aquarium was kept in the same room as the inoculated fish. The 5 control fish were necropsied at the termination of the experiments.

Experiment 1: Serotype Infectivity

Procedures:

Experimental tanks were inoculated with Salmonella concentrations of approximately 100 CFU. The inocula were added directly to the aquaria water containing the resident fish. Aquaria pumps had been operating for three days prior to addition of fish. Adequate oxygenation was provided (8-10 mg/1 DO). Residual chlorine, i.e. $Cl_2(g)$ had likewise been dissipated. The trophic nature of these aquaria in terms of soluble nutrients and resultant biotic productivity feel within the range of normal, oligotrophic parameters. Water temperatures were held within limits of 68°-78°F; pH range was 7.4-8.3; while both free and combined chlorine ranged from 0.0-0.3 mg/1 water (Free and Total Chlorine Test Kit, Moden CN-66, Cat. No. 2231-01, Hach Chemical Co., Ames, Iowa). Five fish were necropsied after 7 days and 14 days residence in the infected water, respectively, unless otherwise noted. Water, as well as fish, were cultured for Salmonella and MPN values, etc. established as discussed previously.

Results:

All environmental conditions (H_2O levels, pH, temperature, etc.) were controlled throughout experimentation. Salmonellae were added directly to tanks housing fish for the previous 5 days. Twelve serotypes, representing 28 strains of Salmonella (see Table A1) showed infective levels of approximately 100 organisms/ml of tank water. Table A1 indicates that salmonellae isolations from viscera at 7 days following inoculation ranged from 80% for cholerae-suis 1103 with an inoculum of 143 cells/ml to 20% for derby 15-4 with 78 cells/ml aquarium water. Serotypes agona, cholerae-suis and typhimurium had greater infectiveness for C. auratus than did serotypes derby and infantis. Differences in infectivity among representative strains of the same serotype were not significant.

All serotypes examined were isolated from fish following water inoculation. Infectivity rates and duration of residence in fish, however, varied from 7 to 14 days. Salmonella typhimurium was the only serotype isolated from fish after 14 days. True colonization, as indicated by greater numbers of salmonellae present in fish viscera as compared to numbers found in environmental water was demonstrable for serotypes cholerae-suis and typhimurium (Table A10).

Experiment 2: Environmental Parameters Involving Infectivity and Transmission of Salmonella

Procedure:

Each aquarium was monitored weekly for levels of chlorine (mg/1) and dissolved oxygen (mg/1). The ph of the water was obtained from 10 ml samples using a Sargent-Welch pH meter (model LS). A 1 liter sample of water from each tank was poured through a suction filter. The grams of suspended material/1 of water was then recorded on the basis of the weight of the dried material collected on the filter paper. In order to increase

the trophic nature of the water, bottom sludge and suspended matter were transferred from the uninoculated, stock fish holding pools to the experimental aquaria to create the desired eutrophic conditions. Aquaria pumps were adjusted to obtain the desired turbidity and a visible, bottom sediment. The rate of water circulation or flow as well as the turbulence was increased by disconnecting the aquaria pump hoses. Nitrogen monitoring, i.e. levels of NH_3 (mg/l) and NO_3 (mg/l), were assessed using chemical kits also from Hach Chemical Company (Low Range Nitrate Test Kit Model NI-14, Cat. No. 14161-00, Ammonia Nitrogen Test Kit, Cat. No. 12524-00).

Results:

Total chlorine (mg/l), pH and temperature values of the water, within the limits of the experiments, did not influence infection rates. The concentrations of suspended matter, however, did influence the rates of infection (Table A1). The grams of suspended material included algae, protozoa and organic debris, i.e., leftover food, fish feces, etc. Salmonellae were added directly to tanks already housing (resident) fish. Aquaria inoculated with S. typhimurium strain Lucus and which contained .01 or greater grams suspended material/ml (eutrophic tanks) showed marked decrease in the number of infected fish at the 7 day period. Eutrophism, therefore, affected the viability of the serotype, and thus its infectivity. Aquaria with .009 or less grams suspended material/ml water (oligotraphic tanks) demonstrated both increased viability of Salmonella and their greater infectivity for goldfish at the 7 day period. In oligotraphic tanks fish were still infected at the 14 day period. No eutrophic tanks contained infected fish at 14 days.

When a detectable, bottom sediment was present, the eutrophic tanks had infectivity values equal to and often greater than the oligotrophic tanks (see Tables A1 and A5). Once a visible sediment was established, Salmonella culture-positive fish were obtained at the 14 day period. Isolations of the organisms from the sediment were also made. In one trial, positive cultures were obtained from both fish viscera and bottom sediment for 6 weeks (Table A1). Studies with S. typhimurium Lucus (Table A1) conducted with 250 salmonellae/ml of water revealed: 60% infection (3/5) at 7 days and no infection (0/5) after 14 days when the suspended material concentration reached 0.021 grams/ml. With the same inoculum, 0.021 grams suspended material/ml and a bottom sediment, the pathogen survived in fish for 5 weeks both 80% infections (4/5). Culture of the sediment was also positive for salmonellae after 5 weeks.

Adequate oxygenation (8-10 mg/l DO) was maintained by the use of Little Giant aquaria pumps and hoses fixed to the side of the aquaria to circulate the water. The rate of flow of the water could be doubled (from 6.9 cm/sec. to 14.2 cm/sec.) by removing the hoses and allowing the pumps to continue circulating the water. In both oligotraphic and eutrophic situations, viability and resultant infections of C. auratus were decreased as water flow and turbulence increased. The results of these experiments are summarized in Table A6.

Table A7 summarizes an analysis of nitrogen content as ammonia (NH_3 mg/l) and nitrate NO_3 mg/l) to indicate the relationship of nitrogen to viability of salmonellae and resultant infection rates. Nitrogen had no significant influence on infection in the experiments conducted. Correlation between nitrogen levels, Salmonella viability and the agents' infectivity could not be ascertained.

Experiment 3: Infective Levels of Salmonella Under Controlled Conditions of Stress

Procedure:

Differences in infection rates as a result of direct, physiological stress were evaluated by either adding fish to the tanks before (unstressed) or after (stressed) inoculation of the aquaria with the various serotypes. "Unstressed" fish resided in the tanks 5 to 7 days prior to inoculation of the tank with salmonellae. "Stressed" fish were allowed to acclimate to the water temperature in floating plastic bags for thirty minutes before being relased into the previously inoculated water.

Further trials involved the addition of 15, noninfected fish to the tanks containing 5 fish (resident fish) which had been kept in the inoculated water for 7 days (see Figure 1 this Section). Necropsies of the 15 additions were performed after 1 day, 5 days and 7 days following exposure to the infected fish. Fish identification was established by clipping one or both caudal fins of the additions.

In another set of trials the infected fish were removed after 7 days and transferred to a clean, uninoculated tank prior to the addition of the 15 new fish mentioned above. These tanks, in one half of the trials, did not contain fish at the time the infected fish were added (see Figure 2 this Section). In the other half of the trials, the tanks also contained 5 "contact" fish. The infection rates for both contact and infected fish, and MPN water levels were ascertained at the 14 day period. All experiments were terminated after 14 days.

Results:

Fish acclimated to the aquaria before almonellae inoculation, i.e. unstressed (Table A4), showed an averaged infection level of 50% at 7 days. After 14 days in 58 trials only 5 trials were unstressed fish were positive for Salmonella. When the fish were added directly after pathogen inoculation of the tanks, i.e. stressed, infections were demonstrable in at least 1 fish in 19 of 21 trials at 14 days, respectively. In both oligotrophic and eutrophic tanks the total number of fish infected increased markedly at the 7 day period when the fish were "stressed" as compared to "unstressed" fish.

Detectable Salmonella concentrations in the water under these same conditions closely parallel this trend. Table A1 indicates that only those tanks housing stressed fish harbored Salmonella detectable at 14 days (based on MPN analysis). Water in all eutrophic tanks at 14 days, regardless of whether or not the fish were stressed, remained negative

for Salmonella. Infections were not present after 14 days. When bottom sediment was detected, this situation was reversed. Infection of fish was demonstrated in eutrophic tanks at 14 days when a bottom sediment was present. MPN levels of salmonellae in water were negative. Culture of sediment at 14 days did reveal viable salmonellae.

Table A9 demonstrates the effect of removing at 7 days (Figure 2) post-inoculation those infected fish initially housed in the experimental tanks. Both stressed and unstressed fish removed to clean, uninoculated water (Figure 2) showed decreased infections (from 80% to 20%) at the end of the 14 day, experimental period as compared to those fish that remained in the original, contaminated tank water.

Data in Table A1 show that those 7 day, infected fish removed to clean tanks which also contained fish (contact fish) failed to infect these fish later as a consequence of fecal shedding (Figure 2). In no case was excretion by the infected fish sufficient to transmit infection to contact fish. In one instance, however, with S. typhimurium strain Lucus at the 170 cell/ml level (with transfer of stressed fish to contact tanks) an MPN determined concentration of Salmonella at 0.013 cells/ml was established in the contact pool. Shedding was taking place.

Additional trials involved the addition of 15 uninfected fish (stressed) to aquaria with 7 day, post-inoculation water (see Figure 1). Tables A1 and A9 show that these fish were infected when less than 1 Salmonella per ml of water was present (based on MPN determinations).

The fish shed the organisms in numbers directly dependent upon the survival ability of the salmonellae in the water and upon the physiological status, i.e. stressed or unstressed condition of the fish present. As the aquatic environment containing the Salmonella becomes less favorable for bacterial survival, e.g. eutrophic conditions, the infections decrease in the fish. Table A8 demonstrates that, over a 14-day experimental period, aquaria which contain no fish show decreases in detectable Salmonella water concentrations as compared to those aquaria with identical water conditions but containing infected fish.

In all conditions analyzed, the continued infection in fish constitutes the important element for persistence of Salmonella in the environment. This is analogous to the need for fecal shedders to be present in higher chordate populations in order to maintain enzootic salmonellosis. A cycle of reinfection takes place and is then augmented under conditions inducing stress in the fish. On the other hand, when detectable, bottom sediment is present, salmonellae will survive independent of the presence of infected fish.

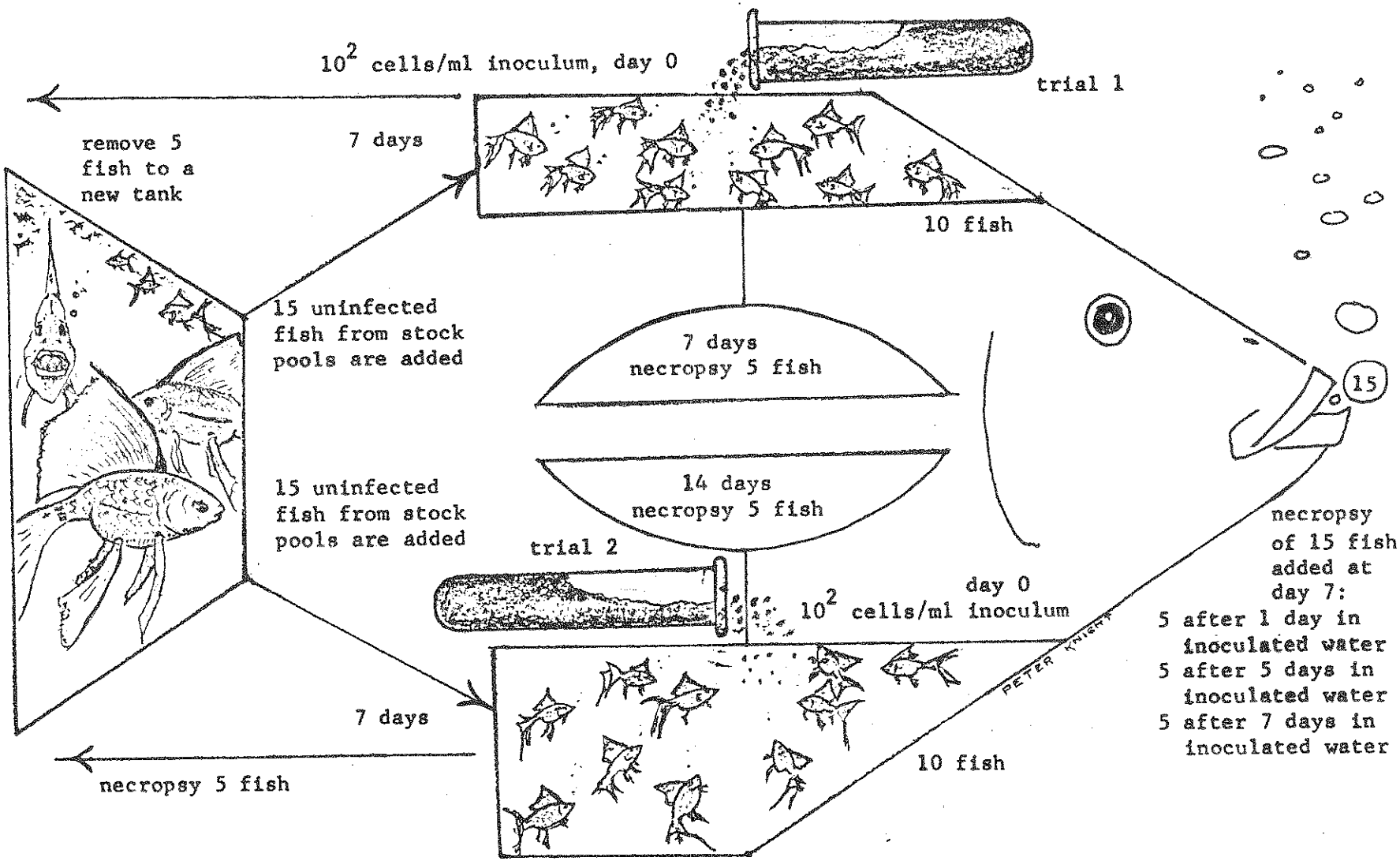


Figure 1: Experiments involving addition of 15, uninfected fish to inoculated pools.

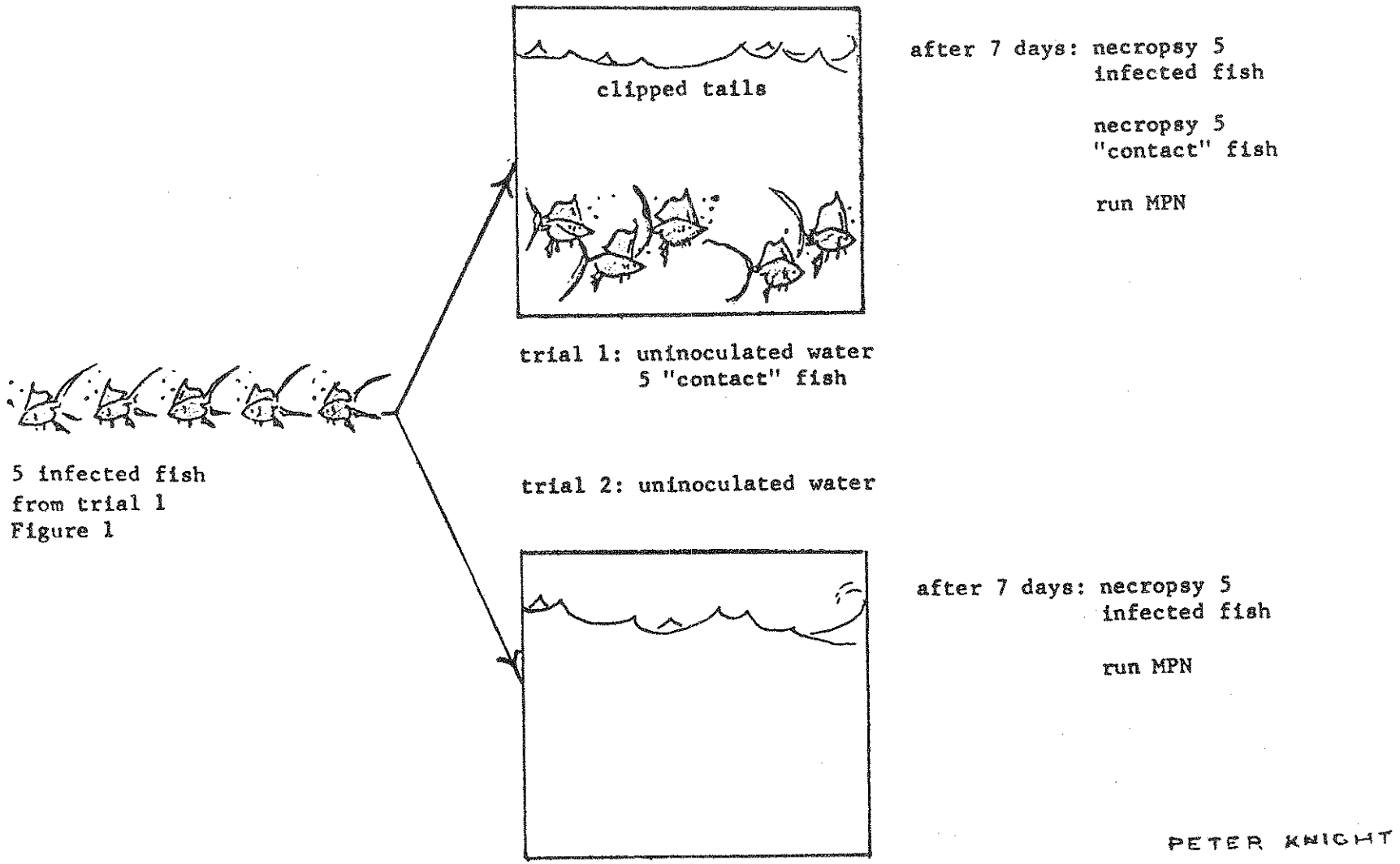


Figure 2: Transfer of infected fish to "contact" pools.

Experiment 4: Rapid Passage of S. agona

Procedure:

To determine whether or not the virulence of Salmonella was altered as a result of residence in fish, a rapid passage of S. agona strain 400 was undertaken. The serotype was added to an aquarium (10^2 cells/ml) containing 10 normal fish. After 7 days the fish were necropsied and cultured for Salmonella. The isolate, designated S. agona 400-R_1, was added at the 10^2 ml level to another aquarium containing 10 fish. Contact was maintained with these fish for 7 days. Strain 400 thus received 4 consecutive transfers in C. auratus at 7 days intervals. A representative of each successive passage was kept as a stock culture on TSA slants. These stocks, i.e. original and passage 1-4 (R_1-R_4) were cultured in TSB for 8 hours to provide inocula. In the first trial, a Salmonella concentration of 10^2 cells/ml was established in each tank housing 15 normal fish. These fish were unstressed as described in Experiment 3. In the second trial, 15 normal fish were added directly to the aquarium after water inoculation with the S. agona cultures strains 400 and R_1-R_4. These fish were categorized as stressed. Five fish were necropsied and cultured as described previously, after 3, 5 and 7 days respectively following contact with the various passages of S. agona 400.

Results:

Further significance of stress on fish and concurrent persistence of Salmonella infection as well as the possible increased numbers of the organism in surface waters was demonstrated following rapid passage of S. agona 400 strain. This strain was rapidly transmitted through serial passages and isolated. Four consecutive, weekly serial passages (as described in Procedures this Section) were made. The procedure was used to ascertain the possible increase or decrease in virulence of the S. agona (see Table A2).

At three days, following water inoculation, infection rates increased as a result of rapid passage. "Unstressed" fish exposed to water containing the fourth passage (R_4) S. agona 400 showed 100% infection. Fish exposed to the third passage (R_3) salmonellae showed only 80% infection. Data from Table A2 show that a progressive increase in infections took place with each successive passage. However, when the fish were added to the tanks directly after (stressed) inoculation of the water, the rapid transfer effect was nullified, and infections remained at high levels (80-100%) for the entire 14 day period.

From the standpoint of "stressed" individuals and increased fecal shedding, it would appear that active colonization had occurred. Salmonella replicated in the fish viscera and were found there in greater numbers than were found in the environmental water. These data are based on the direct plating of viscera and MPN's of the water over the course of experimentation (Table A9). In some of the infected fish, mucoid, hemorrhagic viscera were observed.

Experiment 5: Pathogenicity of S. typhisuis for C. auratus

Procedure:

Investigations involving oligotrophic water were conducted with stressed and unstressed fish as previously delineated in Experiment 3. An initial concentration of 10^5 cells/ml S. typhisuis (NAR) was required to obtain infection with unstressed fish. The TSB with 500 ug/ml nalidixic acid was employed as the enrichment isolation broth in these trials. This serotype does not grow luxurantly in Sel or Tet broths. The S. typhisuis strains used had been laboratory adapted to 500/ug/ml nalidixic acid. Sub-cultures from the broth were plated to Bg and He after 24 and 48 hours incubation at 37°C. These plates were then incubated for 48 and 72 hours. S. typhisuis does not grow rapidly on either Bg or He media.

Results:

Experimentation with nalidixic acid resistant (500 ug/ml) S. typhisuis strains has demonstrated that this serotype can be isolated from fish viscera. This serotype is swine, host-adapted and of low pathogencity except for 3 - 12 week old piglets. Table A3 summarizes these experiments. In "unstressed" fish the infective level was about 10,000 cells/ml. The infection was limited to 3 days post-inoculation. Under "stressed" conditions, infections in fish were established with as few as 100 cells/ml. Isolation of typhisuis was also limited to 3 days post-inoculation. With water concentrations of 10,000 cells/ml, 100% of the fish were positive at 7 days, and, in one case, 20% were positive at 14 days post-inoculation. Salmonella could not be detected in the water at 7 days. Direct plating of fish viscera from both strains 1784 and 1342 revealed 5 to 10 organisms/plate at the same 7 day period (approximately 0.1 gm tissue). This finding represented an actual short term colonization in the fish. The data serve to demonstrate that even an almost apathogenic serotype, typhisuis, has the capability underproper conditions, to colonize in fish.

Status of Control Fish in Section 7

During this study, all control, uninoculated fish were negative for Salmonella except for 1 experiment involving S. typhimurium strain Lucus conducted at the 1100 cell/ml level. Uninoculated control fish kept in the same room were positive at the termination of the trial for Salmonella. The agent which was isolated was believed to be the same strain as that used in the experiment. This conclusion was based upon serological and antibiogram profiles. The accidental infection of the control fish pool (5 fish) cannot be easily explained. Several possibilities for error exist: use of a contaminated fish net; contamination of control fish during necropsy of both control and infected fish; mislabeling of culture flasks or contamination of control aquarium at the time of experimental tank inoculation. This experience does serve to stress that very few Salmonella may be capable of infecting freshwater fish under the right conditions.

Summary and Conclusions:

All 12 serotypes of Salmonella and their respective 32 strains were capable of infecting fish. In tanks of similar water quality and containing essentially the same inocula , differences in infection rates were discernable. These differences reflected virulence patterns established for the same serotypes infecting man and other animals, i.e. cholerae-suis, and typhimurium had the highest infection rates. Adding the fish to the tank after inoculation of the water with salmonellae induced conditions of physiological stress. Under such conditions all serotypes were equally infective.

Increased fecal shedding of salmonellae was correlated with increased infection rates established under conditions of stress. The persistence of Salmonella in surface water without the presence of fish was less than 7 days. Greater numbers of salmonellae were present in both tank water and fish viscera at 7 days when water inoculation was made after the fish had been acclimated, i.e. unstressed.

Increased eutrophy of the water was correlated with decreased infection rates. This situation was reversed when a bottom sediment was established in the tanks. Fish were found to harbor the pathogens for 6 weeks following water inoculation when bottom sediment was present.

Duration of infection is dependent upon water conditions and the physiological status of infected fish. Transfer of infected fish from "infective" to clean water showed decreased infection rates 7 days later. Infection rates were much higher for fish that remained in the inoculated water. A cycle of reinfection was taking place as well as prolongation of the infection.

Fish do constitute an important element for the survival of salmonellae in water. As the aquatic environment becomes less capable of supporting salmonellae, the fish show decreased retention of the pathogen in their viscera.

Fish in nature are continually subjected to conditions potentially capable of producing physiological stress. Salmonellae resident in either bottom sediment or contaminated runoff to their ecosystem pose a threat of increased infections among native fishes. Fish which harbor salmonellae may show increased fecal shedding of the organism as a result of stress. Such fish may serve as "retrospective monitors" of Salmonella in the aquatic environment. This is a result of colonization in the viscera for several weeks following exposure to a point source of pollution in the environment. Active colonization and replication of the pathogen in fish viscera is a major factor in the persistence of Salmonella in the aquatic ecosystem. Water quality and safety standards for freshwater based upon salmonellae concentrations in surface water may indicate present and active pollution or may be retrospective, i.e. previous fecal pollution with salmonellae carriers in the fish population. In any event, whether the Salmonella are entering the system by 1) ongoing contamination or 2) are a result of growth in bottom sediment or 3) are

due to fecal shedding by infected fish is really a moot question. *Salmonella* are human and animal pathogens. Their presence signifies a potential health risk, regardless of their source and point-in-time entry into the aquatic ecosystem.

REFERENCES

1. Hendricks, C.W. Enteric Bacterial Metabolism of Stream Sediment Eluates, Can. Jour. Microbiol., 17:551, 1971.

2. Hendricks, C.W. Increased Recovery Rate of Salmonella from Stream Bottom Sediments vs Surface Waters, Appl. Micro., 21:379-380, 1971.

3. Morse, E.V., Greenwood, D.E., Meyers, E.P., Anderson, V.L., and Duncan, M.A. Experimental Salmonella Infections in Crassius auratus (Goldfish), J. Environ. Sci. Health, A13(4), 325-335, 1978.

4. Battiaux, R. Salmonella Problems in the Sea, pp. 503-519, 1961, in Borgstrom, G. (ed.) Fish as Food, Academic Press, Inc., New York, New York,

5. Hendricks, C.W., and Morrison, S.M. Multiplication and Growth of Selected Enteric Bacteria in Clear Mountain Stream Water, Water Rest., 1:567-576, 1967.

6. Krieger, D.J., and Bond, J.H. Survival of Salmonellae and Fecal Coliforms in Swine Waste Lagoon Effluencts, Clemson University, Clemson, S.C., Abstracts of the Annual Meeting, Water Pool., 1974.

7. Morse, E.V. and Duncan, M.A. Salmonella as Monitors of Fecal Pollution in the Aquatic Environment, Jour. Environ. Sci. Health, Part A, 11(10).

8. Smallbeck, D. and Bromel, M. Relationships Between Bacterial Populations in Bottom Sediments and Overlying Water in a Farm Animal Waste Lagoon, North Dakota State University, Fargo, Abstracts of the Annual Meeting, Water Poll. p. 52, 1974.

9. Kleeman, I., Frank, S., Abrahamson, A.E. Food Poisoning Outbreaks Involving Smoked Fish--Their Epidemiology and Control, Amer. J. Public Health, 32:151-158, 1942.

10. MacKenzie, G.R., Livingston, D.J. Salmonellae in Fish and Food, S. Afr. Med. J., 42:999-1003, 1968.

11. Shervan, J.M., Liston, J. A Review of Food Poisoning Caused by Fish and Fishery Products, J. Appl. Bact., 18:522-534, 1955.

12. Huschman-Brunner, G.H. Experiments on the Possibilities and Course of Infections with Salmonella enteriditis and Salmonella typhimurium in Freshwater Fish, Zbtl. Bakd. Hyg. I Abt., Orig. B, 158:412-431, 1974.

13. Martin, W.T. Occurrence and Survival of Salmonella in the Alimentary Tract of Some Fresh Water Fishes, M.S. Thesis, Kansas State University, Manhattan, 1966.

14. Morse, E.V., and Duncan, M.A. Salmonellosis An Environmental Health Problem, Jour. Amer. Vet. Med. Assoc., 165:1015-1019, 1974.

15. Morse, E.V. and Duncan, M.A. Salmonellosis An Environmental Problem Affecting Man and Animals, Proc. 78th Ann. Mtg., U.S. Ani. Hlth. Assoc., 288-299, 1974.

16. Lewis, D.H. Retention of Salmonella typhimurium by Certain Species of Fish and Shrimp, J. Amer. Vet. Med. Assn., 167:551-552, 1975.

Appendix - Section 7
Tables A1 - A10

Table A1. Infection of C.auratus by Salmonella serotypes.

Serotype	Inoculum (cells/ml)	MPN cells/ml Day 0	Day 7	Day 14	pH	Chlorine mg/l free	total	Temp. °F	pre/post*	new/exp**	Infection (% of necropsy) 3day	7day	14day	Post 1 week*** 1day	5day	7day	Hoses on/off	Contact fish pos. or neg. for Salmonella	Water Conditions
agona																			
400	150	160	.03	0	7.9	.1	.3	74	post	exp	-	66^{+}_{+}	0	20	0	0	on	-	clear
"	150	50	.13	0	7.8	.1	.4	74	pre	exp	-	0^{+}_{+}	0	0	0	0	on	-	clear
"	150	160	0	0	7.8	.1	.2	74	pre	new	-	40^{+}_{+}	0	0	0	0	on	neg	clear
"	150	50	0	0	7.9	.1	.3	74	pre	exp	-	0^{+}_{+}	0	0	0	0	on	-	clear
agona																			
400 R_1	204	24	.004	0	8.2	0	.1	74	pre	new	-	20^{+}_{+}	0	0	0	0	on	neg	clear
"	141	90	.002	0	7.7	0	.1	78	pre	new	-	33^{+}_{+}	0	0	0	0	on	neg	clear
"	141	160	.007	0	7.6	0	.1	78	post	new	-	50^{+}_{+}	0	0	0	0	on	neg	clear
anatum																			
200	9.6	8	0	0	7.8	0	.1	72	pre	new	-	20^{+}_{+}	0	0	0	0	on	neg	clear
"	9.6	13	0	0	8.0	0	.1	74	pre	exp.	-	0^{+}_{+}	0	0	0	0	on	-	clear
"	240	160	0	0	8.0	0	.1	74	pre	exp	-	10^{+}_{+}	0	-	-	-	on	-	clear
"	240	160	0	0	8.1	0	.1	74	pre	exp	-	0	0	-	-	-	on	-	clear
"	133	160	0	0	8.3	0	.2	72	pre	exp	-	0	0	-	-	-	on	-	clear
"	133	160	0	0	8.1	0	.2	75	pre	exp	-	0	0	-	-	-	on	-	clear
anatum																			
17	147	160	0	0	8.1	0	.1	72	pre	new	-	40	0	0	0	0	on	neg	clear
"	147	30	0	0	8.2	0	0	75	pre	exp	-	20	0	0	0	0	on	-	clear
"	203	90	0	0	8.2	0	0	73	pre	exp	60	40	0	0	0	0	on	-	clear
derby	78	50	0	0	8.1	0	0	72	pre	exp	-	0	0	0	0	0	on	-	clear
"	78	50	0	0	7.9	0	0	74	pre	exp	-	20	0	0	0	0	on	-	clear
eimsbuettel	210	30	0	0	7.8	0	0	74	pre	exp	60	40	0	0	0	0	on	-	clear
"	210	60	0	0	7.8	0	0	75	pre	exp.	20	0	0	0	0	0	on	-	clear
enteriditis																			
NAR	127	90	0	0	8.1	0	0	76	pre	exp	0	0	0	0	0	0	off	-	clear
Ostenberg	153	90	0	0	8.1	0	0	76	pre	new	40	20	0	0	0	0	off	neg	clear
25	58	50	0	0	7.9	0	0	76	pre	exp	40	40	0	0	0	0	off	-	clear
infantis	100	90	0	0	7.7	0	0	76	pre	exp	0	0	0	0	0	0	off	-	clear
"	100	50	0	0	7.9	0	0	74	pre	exp	20	0	0	0	0	0	on	-	clear
montivideo	96	23	0	0	7.9	0	.1	76	pre	exp	60	0	0	0	0	0	off	-	clear
"	96	90	0	0	7.8	0	0	74	pre	exp	60	40	0	0	0	0	on	-	clear

Table A1,cont.

typhimurium																			
Lucus	170	160	.9	1.6	7.8	0	.1	74	post	new	-	100	100	100	100	100	on	neg++	clear
"	170	160	.3	.3	8.0	0	.2	76	post	new	-	100	100	100	100	100	on	-	clear
"	1100	700	0	0	8.0	.2	.25	74	pre	exp	-	0	0	0	0	0	off	-	.0056+++
"	1100	380	0	0	8.0	.1	.3	70	pre	exp	-	0	0	0	0	0	off	-	.0034
"	1100	700	0	0	8.0	0	0	76	pre	exp	-	0	0	0	0	0	off	-	.0001 +++
"	106	90	0.2	0	7.4	0	0	76	pre	exp	-	40	20	-	-	-	on	-	sludge
"	106	50	0.8	0	7.4	0	0	76	pre	exp	-	20	40	-	-	-	on	-	sludge(+3 wks)
"	106	90	0	0	7.5	0	0	74	pre	exp	-	40	40	-	-	-	on	-	sludge(+6 wks)
"	135	160	0	0	7.5	0	0	74	pre	exp	-	20	0	0	0	0	on	-	.0750
"	135	160	0	0	7.8	0	0	76	pre	exp	-	0	0	0	0	0	on	-	.0690
"	135	160	1.3	0	7.4	0	0	74	pre	exp	-	60	0	20	0	0	on	-	.0035
"	135	90	0	0	7.4	0	0	76	pre	exp	-	0	0	0	0	0	on	-	.0700
"	164	30	0	0	7.8	0	0	72	pre	exp	-	20	40	80	0	0	on	-	.0004
"	164	24	.0013	0	8.1	0	0	72	pre	new	-	20	0	0	0	0	on	-	.0026
"	164	30	0	0	7.7	0	0	70	pre	exp	-	0	0	0	0	0	off	-	.0175
"	164	50	.002	0	7.7	0	0	74	pre	exp	-	60	60	80	60	60	on	-	sludge
"	164	24	0	0	7.9	0	0	72	pre	exp	-	0	0	0	0	0	on	-	.0800
"	164	160	0	0	7.8	0	0	72	pre	exp	-	0	0	0	0	0	on	-	.2099
"	164	24	0	0	8.0	0	0	72	pre	exp	-	20	0	20	0	0	on	-	clear
"	164	24	0	0	7.9	0	0	72	pre	new	-	60	0	60	0	0	on	neg	clear
"	164	24	0	0	7.9	0	0	72	pre	exp	-	20	0	0	0	0	on	-	clear
"	164	24	0	0	8.0	0	0	72	pre	exp	-	0	0	0	0	0	off	-	clear
"	356	160	0	0	7.8	0	.1	72	pre	exp	-	40	0	-	-	-	on	-	clear
"	356	160	0	0	7.8	0	.1	72	post	exp	-	80	80	-	-	-	on	-	clear
"	150	160	0	0	7.8	0	0	72	pre	exp	sludge + for Salmonella - 6 weeks,fish-6 weeks- 100% infected								
"	250	160	0	0	7.3	0	0	76	pre	exp	-	20	0	-	-	-	on	-	.0350
"	250	160	0	0	7.3	0	0	76	post	exp	-	80	0	20	0	0	on	-	.0350
"	250	160	0	0	7.4	0	0	70	pre	new	-	40	0	0	0	0	on	neg	.0340
"	250	160	0	0	7.4	0	0	70	post	new	-	80	0	0	0	0	on	neg	.0340
"	250	160	.23	0	7.5	0	0	78	pre	exp	-	60	0	-	-	-	on	-	.0210
"	250	250	.23	0	7.5	0	0	78	post	exp	-	100	80	40	0	0	on	-	.0210(sludge, and fish (80%) positive 5 weeks)
typhimurium																			
16B	77	24	5	.002	7.9	0	.1	74	post	exp	-	100+	100	100	100	100	on	-	clear
"	77	24	5	.002	7.8	0	.1	74	post	exp	-	100	100	100	100	100	on	-	clear
"	7.7	2.3	0	0	7.8	0	.1	76	post	exp	-	50+	16.7	100	80	80	on	-	clear
"	84	90	.13	.05	7.9	0	0	76	post	new	-	100+	0	0	40	0	on	neg	clear
"	84	60	.17	.05	8.0	0	.1	74	post	new	-	80+	0	60	40	40	on	neg	clear

Table A1, cont.

typhimurium																			
var. Copenhagen																			
Rodruck	45.8	9	.013	0	7.9	0	.1	76	post	exp	-	100+	16.7	-	-	-	on	-	clear
"	45.8	9	.013	0	7.8	0	.1	76	post	exp	-	100+	16.7	-	-	-	on	-	clear
"	4.58	4	0	0	7.8	0	.1	76	post	exp	-	100+	66	-	-	-	on	-	clear
"	.458	.04	.0017	0	7.8	0	.1	76	post	exp	-	83+	16.7	-	-	-	on	-	clear
Talkington	216	160	3.1	.023	7.8	0	0	78	post	exp	-	100+	33	66	83	0	on	-	clear
"	216	160	3.1	.03	7.8	0	0	78	post	exp	-	100+	33	16.7	50	33	on	-	clear
"	21.6	24	.5	.007	7.6	0	0	78	post	exp	-	66+	33	-	-	-	on	-	clear
"	21.6	5	.5	.02	7.6	0	0	78	post	exp	-	66+	66	-	-	-	on	-	clear
cholerae-suis																			
1102	127	44	0	0	7.8	0	0	72	pre	exp	60	0	0	-	-	-	on	-	clear
2992	121	44	0	0	7.6	0	0	72	pre	exp	40	0	0	-	-	-	on	-	clear
1103	143	124	0	0	7.9	0	0	74	pre	exp	100	80	20	-	-	-	on	-	clear
164	51	20	0	0	7.9	0	0	74	pre	exp	40	20	0	-	-	-	on	-	clear
var. Kunzendorf																			
local pig	67	22	0	0	7.8	0	0	72	pre	exp	80	20	0	-	-	-	on	-	clear
3648	43	24	0	0	7.6	0	0	74	pre	exp	40	40	0	-	-	-	on	-	clear
3094	83	50	0	0	7.7	0	0	74	pre	exp	40	20	0	-	-	-	on	-	clear
3052	100	50	0	0	7.7	0	0	74	pre	exp	80	60	0	-	-	-	on	-	clear

* Fish were added before(pre) or after(post) water inoculation with Salmonella, i.e. pre = unstressed and post = stressed.

** After one week of residency in inoculated water, infected fish were either removed to a new tank(new) or allowed to remain in the experimental tank (exp).

*** After one week of experimentation, 15 new,uninfected fish were added to the tanks. Five fish were necropsied after 1 day, 5 days and 7 days, respectively.

\+ Infection based on necropsy of 6 fish, i.e. 16.7% = 1/6 positive. All other % infection is based on necropsy of 5 fish,i.e. 20% = 1/5 positive.

++ Contack fish water had an MPN value after 7 days occupancy by infected fish of .013 cells/ml water.

+++ Grams suspended material/ml tank water, sludge = visible bottom sediment, clear = 0 grams/ml water.

MPN 5 tube, 3 fold dilution technique(Standard Methods for the Examination of Water and Wastewater, 13 ed.)

Table A2. Increased Virulence of S. agona 400 with rapid passage.

Passage	Inoculum (cells/ml)	Fish added pre or post inoculation of water	Infection (100% = 5/5) 3 day	7 day	14 day
Original	140	pre	40	0	0
Original	140	post	100	80	80
R_1	189	pre	40	20	0
R_1	189	post	80	80	80
R_2	146	pre	60	60	0
R_2	146	post	100	80	80
R_3	195	pre	80	20	0
R_3	195	post	100	80	100
R_4	219	pre	100	20	0
R_4	219	post	100	80	100

R_1 = First passage of S. agona 400 through fish (7 days).

R_2 = Second passage, i.e. R_1 first passage through fish.

R_3 = Third passage, i.e. R_2 first passage through fish.

R_4 = Fourth passage, i.e. R_3 first passage through fish.

pre = unstressed

post = stressed

Table A3. Experimental infection with S. typhisuis (NAR),

Strain	Inoculum (cells/ml) CFU	MPN* (cells/ml) 1 day	7 day	fish added pre or post inoc. of water	Infection 3 day	7 day	14 day
3198	58	0	0	pre	0	0	0
3198	58,000	0	0	pre	100	0	0
3198	136	160	0	post	40	0	-
3198	13,600	16,000	0	post	100	100	-
1784	64	0	0	pre	0	0	0
1784	64,000	.08	0	pre	60	0	0
1784	158	160	0	post	80	0	-
1784	15,800	16,000	0	post	100	100	-
2637	43	0	0	pre	0	0	0
2637	43,000	.07	0	pre	100	0	0
2637	115	160	0	post	40	0	-
2637	11,500	16,000	0	post	100	100	-
1342	30	0	0	pre	0	0	0
1342	30,000	.07	0	pre	100	0	0
1342	110	90	0	post	80	0	-
1342	11,000	16,000	0	post	100	100	20

* MPN = most probable number

All % infection based on necropsy of 5 fish i.e. 100% = 5/5

pre = unstressed

post = stressed

Table A4. Influence of stress on infection of C. auratus with various strains of Salmonella.

Serotype	Inoculum cells/ml	Water** conditions	Fish added pre(unstressed) post(stressed) water	7 day transfer to new tank (+) or (-) inoculation	% Infection+ 7day	14day	Post 7 days* 1day	5day	7day
agona 400	150	clear	unstressed	+	0 ++	0	0	0	0
"	150	clear	stressed	-	66 ++	0	20	0	0
" R_1	140	clear	unstressed	+	33 ++	0	0	0	0
" R_1	140	clear	stressed	+	50	0	0	0	0
typhimurium									
16B	84	clear	stressed	+	80 ++	0	60	40	40
"	77	clear	stressed	-	100 ++	100	100	100	100
"	7.7	clear	stressed	-	50 ++	16.7	100	80	80
Lucus	170	clear	stressed	+	80	0	0	100	80
"	170	clear	stressed	+	80	0	100	60	100
"	150	clear	stressed	-	80	80	-	-	-
"	150	clear	unstressed	-	40	0	-	-	-
"	250	.0350	unstressed	-	20	0	0	0	0
"	250	.0350	stressed	-	80	0	20	0	0
"	250	.0210	unstressed	-	60	0	0	0	0
"	250	.0210	stressed	-	100	20	40	0	0
"	250	.0350	unstressed	+	40	0	0	0	0
"	250	.0350	stressed	+	80	0	0	0	0
var. Copenhagen									
Rodruck	46	clear	stressed	-	100 ++	16.7	-	-	-
"	.46	clear	unstressed	-	83 ++	16.7	-	-	-
"	46	clear	stressed	-	40	0	-	-	-

* After 7 days, 15 uninfected fish were added to experimnetal tanks.

** Grams suspended material/ml water, clear = o grams/ml. ++ Infection based on necropsy of 6 fish.

\+ Infection based on necropsy and culture of 5 fish, i.e. 20% infected = 1/5 positive for Salmonella.

Table A5. Decreased Salmonellae infection rate as a result of transferring fish to an uninoculated tank at 7 days experimentation.

Serotype	Inoculum (cells/ml)	Infection 7 day	Infection 14 day	7 day transfer* + or -	Water Conditions
typhimurium					
16B	84	100	0	+	clear
"	84	80	0	+	clear
"	77	100	100	-	clear
"	77	100	100	-	clear
typhimurium					
Lucus	170	80	0	+	clear
"	170	80	0	+	clear
"	106	20	40	-	clear
"	106	40	40	-	clear
"	164	20	0	+	.0026**
"	164	20	40	-	.0005
"	124	60	60	-	sludge**
"	250	60	20	-	sludge
"	164	60	0	+	sludge
"	106	40	40	-	sludge
"	106	20	40	-	sludge
"	250	20	0	-	.0300
"	250	80	0	-	.0300
"	250	40	0	+	.0300
"	250	80	0	+	.0300
"	250	100	20	-	.0300

* At 7 days post inoculation of experimental tanks, the fish were either removed to an uninfected tank or allowed to remain in the infected water.

** grams suspended material/ml tank water
sludge = visible, bottom sediment, clear = 0 grams/ml

All % infection based on necropsy of 5 fish i.e. 100% = 5/5

Table A6. The effect of water flow rate on the infection of *C. auratus* to *Salmonella* serotypes.

Serotype	Inoculum cells/ml	Water* conditions	Infection(100% = 5/5) 7 days	14 days post 1day	7 days** 5day	7day	hoses+ on/off
eimsbuettel	210	clear	40	0	0	0	0	on
"	210	clear	0	0	0	0	0	off
enteriditis 35	58	clear	40	20	0	0	0	off
enteriditis Ostenberg	153	clear	40	20	0	0	0	off
infantis	100	clear	20	0	0	0	0	on
"	100	clear	0	0	0	0	0	on
typhimurium Lucus	170	clear	80	0++	100	60	100	on
"	1100	.0056	0	0	0	0	0	off
"	1100	.0034	0	0	0	0	0	off
"	1100	.0034	0	0	0	0	0	off
"	1100	clear	0	0	0	0	0	off
"	1100	clear	0	0	0	0	0	off
"	106	clear	40	40	0	0	0	on
"	106	clear	40	20	0	0	0	on
"	164	.0045	20	40	80	0	0	on
"	164	.0175	0	0	0	0	0	off
"	164	sludge	60	60	80	60	60	on
"	164	.0080	0	0	0	0	0	on
"	164	.2099	0	0++	0	0	0	on
"	164	clear	60	0++	60	0	0	on
"	164	clear	0	0	0	0	0	off

* Grams suspended material/ml, sludge = visible sediment, clear = 0 grams/ml water.

** At one week after water inoculation, 15 uninfected fish were added to each of the experimental tanks. 5 fish from each tank were necropsied and cultured after 1 day, 5 days,and 7 days, respectively.

\+ By removing the aquaria pump hoses, the rate of water flow doubled.

++ Infected fish, after 7 days, were transferred to another uninoculated tank. These fish were necropsied and cultured 7 days later.

All % infection was based on necropsy of 5 fish, i.e. 100% = 5/5 inf.

Table A7. Influence of nitrogen content (total) on infection of C. auratus to Salmonella.

Inoculum cells/ml	Grams suspended material/ml water	Day 1 NH_3 (mg/l)	Day 7 NH_3 (mg/l)	Day 1 NO_3 (mg/l)	Day 7 NO_3 (mg/l)	Infection (% of 5 fish) Day 7	Day 14	Additional Information
S.typhimurium Lucus								
135	.0750	1.0	-	4.0	-	20	0	
135	.0690	0.5	-	8.5	-	0	0	
135	.0035	0.5	-	5.0	-	60	40	
135	.0700	1.0	-	0.8	-	0	0	
164	.0045	-	-	7.0	3.5	20	40	
164	.0026	-	-	4.0	1.5	20	0	
164	.0175	-	-	2.0	0.0	20	0	no hose attachment
164	.0500	-	-	5.5	5.5	60	60	visible sediment
164	.0800	-	-	2.8	2.8	0	0	i.e. (sludge)
164	.2099	-	-	1.0	1.0	0	0	
164	.0000	-	-	3.0	3.0	20	0	
164	.0000	-	-	0.2	0.6	60	0	
164	.0000	-	-	0.0	0.4	no fish		
164	.0000	-	-	0.0	0.5	20	0	
164	.0000	-	-	0.0	0.4	0	0	
150	.2010	-	-	0.8	-	sludge pos. for Salmonella for 6 weeks		
250	.0350	0.5	-	0.7	-	20	0	
250	.0340	0.5	-	1.4	-	40	20	
250	.0210	0.5	-	0.1	-	100	20	visible sediment
S.typhimurium 24 RH_2O								
160		0.25	0.50	5.0	5.0	20	0	
160		1.25	0.75	2.0	2.0	20	20	
160		0.50	0.50	4.0	4.0	40	0	

Table A8. Importance of residence in fish to survival of Salmonella in water.

Serotype	Inoculum cells/ml	MPN cells/ml Day0	Day1	Day2	Day4	Day5	Day7	Day14	Water Conditions
		without fish residence							
agona									
400	150	50	9	2.3	0.4	0	0	0	clear
"R_1	140	160	1	.7	.2	0	0	0	clear
"R_1	140	90	1	.9	.2	0	0	0	clear
typhimurium									
Lucus	1100**	700	22	.7	0	0	0	0	.3333*
"	1100**	700	18	.7	0	0	0	0	.0008
"	164	160	17	.23	.2	0	0	0	clear
typhimurium									
24 RH_2O	270	160	.4	.23	.004	0	0	0	clear
		with fish residence							
agona									
400	150	160	-	-	-	-	.03	0	clear
"	150	50	-	-	-	-	.13	0	clear
"R_1	140	90	-	-	-	-	.007	0	clear
'R_1	140	160	-	-	-	-	.002	0	clear
typhimurium									
Lucus	164	160	-	-	-	-	.002	0	.0005
"	164	160	-	-	-	-	.0013	0	.0026
"	164	160	-	-	-	-	.002	0	.0500

* grams suspended material/ml tank water, clear = 0 grams/ml.

** Water flow rate was increased (doubled from 6.9 cm/sec to 14.2 cm/sec) by disconnecting the aquaria pump hoses.

Table A9. Observed necrosis of fish viscera* correlated with isolation of Salmonella from direct plating of 0.01 grams viscera.

Serotype	Inoculum cells/ml	Infection(5 of 5 fish necropsied) 7 days	14 days	Direct plating pos/neg for Salmonella 7 days	14 days	Observed necrosis pos(+) neg(-)
agona						
400R$_1$	140	50	0	pos(2/5)	neg	+
"	140	20**	0	pos(1/5)	neg	-
typhimurium						
Lucus	170	100	100	pos(4/5)	pos(2/5)	+
"	170	100	100	pos(3/5)	pos(1/5)	+
"	250	80	0	pos(1/5)	neg	+
"	250	40++	0++	pos(1/5)	neg	-
"	250	100	20	pos(2/5)	neg	-
typhimurium						
16B	84	100	0	pos(2/5)	neg	-
"	77	100**	100	pos(3/5)	pos(1/5)	+
typhimurium var. Copenhagen						
Talkington	216	80**	20	pos(1/5)	neg	-
cholerae-suis						
1103	143	100	20	pos(1/5)	pos(1/5)	+

* Necrosis = pseudomembranous, bloody viscera.

** Fish deaths observed and posted after 2 days experimentation.

All fish added after water inoculation with Salmonella(stressed) except (++) = unstressed.

Table A10. Direct plating of fish viscera and experimental water

Serotype	Inoculum cells/ml water	7 day MPN cells/ml water	Direct plating of .1 ml tank water	Direct plating of .01 loop of minced viscera from 1 fish
agona 400 R_1	140	0	0	4
typhimurium Lucus	250	0	0	10
"	250	0	0	17
"	250	.23	0	1+
typhimurium 16B	77	5	0	2+
"	84	.17	0	2+
cholerae-suis 1103	143	0	0	14

1+ = greater than 20 cells / plate.

2+ = greater than 100 cells / plate.